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Attorney Docket No. 1418.04 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: MANFREDI et al.,	)	Group Art Unit: 1636
Serial No.: 10/040,964	)	
Filed: January 4, 2002	)	
For: METHOD OF DETECTING EXTRACELLULAR PROTEIN-PROTEIN INTERACTIONS	)	Examiner: D.A. Lambertson

February 3, 2004

Commissioner for Patents
Washington, DC 20231

DECLARATION UNDER 37 C.F.R §1.131

Sir:

I, John Manfredi, hereby declare and state that:

1. I am presently employed by Myriad Genetics, Inc. as the head of Screening Technologies and am responsible for the development of drug screening technologies at Myriad. I have been employed by Myriad at its headquarter in Salt Lake City, Utah, U.S.A., since December 1, 1999.

2. Prior to August 25, 2000, that is, prior to September 29, 2000, I conceived the invention as defined in Claims 65-72, 80, 81, 85, 86, and 90-92. In order to initiate the patent application filing process, I prepared an invention disclosure on the invention on August 25, 2000. A copy of the disclosure is attached hereto as Exhibit A. The invention disclosure was signed by me. It was also signed by Dr. Kirill Ostanin of Myriad as a witness. On August 25, I gave a copy of the invention disclosure shown in Exhibit A to Mr. Chris Wight, the then General Counsel for Myriad, and requested that a patent application be promptly prepared and filed on the invention.

3. On October 2, Mr. Jay Zhang, our first in-house patent attorney started his first day at Myriad, and was instructed to prepare a patent application on the invention.

4. On October 17, 2000, I met with Mr. Jay Zhang to discuss the invention and preparation of a patent application thereupon. Dr. Kirill Ostanin was present at the meeting. As evidence of this meeting, a copy of the notes Mr. Zhang took during the meeting is attached herein as Exhibit B.

5. On October 31, 2000, Mr. Jay Zhang started writing a patent application on the invention. The Microsoft Word document he first created was named "Intein-

based two-hyrid assay.doc" and a printout of the Properties dialog box associated with the document showing automatically updated and maintained statistics of the document is attached as Exhibit C. A copy of the document as of December 13, 2000 is also attached as Exhibit D.

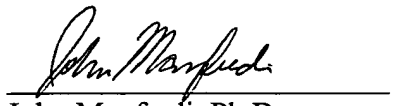
6. On November 9, 2000, Mr. Jay Zhang started preparing drawings for the patent application and created a Microsoft PowerPoint document named "TWO-HYBRID SYSTEM.ppt." A printout of the Properties dialog box associated with the document showing automatically updated and maintained statistics on the document is attached as Exhibit E. A copy of the document as of January 4, 2001 is also attached as Exhibit F.

7. On December 13, 2000, I received a draft patent application from Mr. Jay Zhang for review. The copy is the same as that of Exhibit D. I diligently reviewed the document and returned it to Mr. Jay Zhang after the Christmas Holiday.

8. Upon incorporating my comments, Mr. Jay Zhang promptly filed a provisional application after the New Year's Day. U.S. Provisional Application Serial No. 60/259,759, to which the above-referenced application claims priority was accorded a filing date of January 4, 2001.

9. It is noted for the record that no admission is made or intended as to the exact date of conception except that the conception of the invention defined by the rejected claims was completed prior to August 25, 2000, that is, prior to September 29, 2000. It is also noted for the record that no admission is made or intended as to the existence or date of any actual reduction to practice by the Applicant. However, it is believed that the above data is sufficient to satisfy the requirements under 37 C.F.R. §1.131.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



John Manfredi, Ph.D.

February 3, 2004

Date

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EXHIBIT A

#2

ADRIAN HOSAEN

Intein-Directed Yeast Two-Hybrid Assay (IDY2H)
First Draft, Invention Disclosure

Background

Inteins are defined as protein sequences embedded in-frame within a precursor protein sequence and excised during a process termed protein splicing. This maturation process is a post-translational event involving precise excision of the intein sequence and coincident joining of the flanking sequences, termed exteins, via a peptide bond. For example, in Saccharomyces the protein encoded by *VMA1* is processed by protein splicing to yield an intein-encoded endonuclease and an extein-encoded vacuolar ATPase. Nearly 100 inteins have been identified (http://www.neb.com/neb/inteins.html). Notable features of the autoproteolytic event are these:

1. Processing does not require other molecules. That is, it is autocatalytic.
2. Processing does not require specific extein consensus sequences; indeed, the only extein sequence requirement is that the residue immediately C-terminal to the intein is Cys, Ser, or Thr.
3. Information necessary for the event is contained in sequences at the N- and C-termini of the intein. Thus, deletions that leave the N-terminal ~150 residues and C-terminal ~50 residues of an intein do not affect autoproteolytic processing.

Protein splicing was originally believed to be an intramolecular event. The *Ssp* DnaE protein, however, has recently been demonstrated to arise from *trans*-splicing of a split intein. Thus, two genes encode DnaE in the cyanobacterium *Synechocystis* sp. strain PCC6803. One gene encodes the N-terminal region of DnaE fused to 123 residues that resemble the N-termini of reported inteins; a second gene expresses a fusion protein in which sequences resembling intein C-termini are fused to the remainder of DnaE. Intermolecular protein splicing results in synthesis of active, full-length DnaE with concomittant removal of the two intein fragments.

Utility

The introduction of the two-hybrid assay in 1989 enabled researchers to rapidly and sensitively detect interactions between proteins. The remarkable utility of this assay is indicated by the proliferation of modifications that attempt to circumvent limitations inherent in the original assay. For example, the original configuration of the two-hybrid assay requires that the interacting proteins (or fusions containing them) be transported into the nucleus and functional in the nuclear environment. Efforts to circumvent this requirement has spawned a number of variations of the original assay, including:

1. the "ubiquitin-based split protein sensor;"
2. the "Sos recruitment system" and its successor, the "Ras recruitment system;"
3. the "split DHFR system;"
4. the "β-galactosidase complementation system."

Each of these techniques has limitations that are often unique to the particular system being used and protein-protein interaction being tested. Many limitations are likely attributable to the unpredictable functionality of the fusion proteins that must be engineered for each system. Other limitations stem from the specific nature of the protein-protein interaction being examined and the differing cellular backgrounds and compartments in which those interactions can be tested. In fact, experience within the research community has shown that not one of the four assays listed above can be successfully applied to most members of a protein class, such a membrane proteins. For this reason, it is important to expand the armamentarium of assays that detect protein-protein interactions. The present invention is one such assay, which may be useful in different cellular backgrounds, different cellular compartments, and even and in vitro conditions.

Brief Description of Invention

Proteins (designated X and Y) whose interactions are to be examined are expressed as two separate fusions, each of which contains an intein sequence and part of a selectable or quantifiable protein (i.e., reporter). Neither fusion, in itself, is competent for reporter activity, since it contains only a part of the reporter. Likewise, coincident expression of the two proteins that are not competent for association will fail to give reporter activity. On the other hand, if the two fusion proteins can interact (by virtue of association between X and Y), intein-directed protein splicing will result in generation of functional reporter, allowing detection of the interaction.

Detailed Description of Invention

Plasmids vectors will be constructed that enable expression of two separate intein-containing fusion proteins. One protein will contain the N-terminal intein residues that are necessary for the intein's autocatalytic activity; these residues will be preceded by the N-terminal half of a reporter such as His3p, Ura3p, or β-galactosidase and followed by one of the two proteins whose interaction will be tested. The second fusion protein will contain the C-terminal intein residues necessary for autoproteolytic processing; these will be preceded by the second potentially interacting protein and followed by the C-terminal half of the reporter. Cells transformed with the two expression constructs will be assayed for reporter activity as a readout of protein-protein association.

An illustrative example follows. A chimeric gene will be constructed that encodes a fusion protein consisting of (from the N- to the C-terminus): the N-terminal 154 residues of the 267 amino acid S. cerevisiae Ura3p; 187 N-terminal residues of the VMA1 intein; a flexible linker composed of residues 114 to 163 of IRS1; and residues 31 to 150 fo the PI3 kinase catalytic subunit, p110β. A second chimeric gene will be constructed that encodes a fusion protein consisting of (from the N- to the C-terminus): residues 427-613 of the PI3 kinase regulatory subunit, p85; the IRS1 linker; 50 C-terminal residues of the VMA1 intein; and the C-terminal 113 residues of Ura3p. Each coding sequence will be

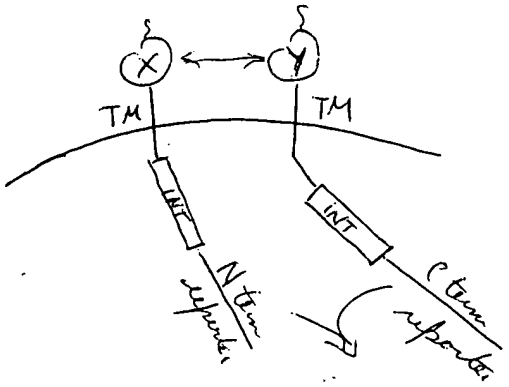
engineered to be expressed from the galactose-inducible GAL1 promoter, and *ura3* yeast will be transformed with plasmids that express the two chimeric genes. Transformants will be tested for galactose-dependent uracil prototrophy, which would indicate association between p110 and p85.

The intein protein-splicing domain consists of about 100 and 50 residues derived from the N- and C-termini, respectively, of the intein. The affinity of these regions for one another is unknown, although current models of the mechanism of transplicing of *Ssp* DnaE postulate substantial affinity, although this may be exceptional. So, it is conceivable that intein-driven association of the fusion proteins may occur independently of an interaction between the tested proteins (i.e. independently of p110 and p85 in the example above). This will be tested using fusion proteins that lack p110 and p85. If indeed signal is observed, mutagenesis will be performed on the intein domains to find mutants that fail to self-associate yet retain autoproteolytic activity. In a complementary approach we will test the advantages of using the split intein from Ssp *dnaE* in place of the VMA1 intein. If association of the Ssp. DnaE intein contributes to unacceptably high background signal (i.e., association of fusion proteins without p110 and p85), the Ssp DnaE intein sequences will be mutagenized, and mutants will be identified that fail to self-associate but exhibit transplicing activity.

Extensions

The autocatalytic nature of intein-directed protein splicing allows use of this invention for detecting interactions between extracellular proteins. Indeed, experience has shown that conventional two hybrid assays are unreliable in detecting interactions between proteins that normally function in an oxidized, extracellular environment. Two minor modifications of the intein-based two-hybrid assay described above may allow study of interactions between extracellular proteins:

1. Each fusion protein is engineered to contain a signal peptide at its N-terminus.
2. The reconstituted reporter functions extracellularly. Extracellular reporters that allow both genetic selection and quantitative assays will be tested.



8.25.00

John Manfredi
8.25.00

EXHIBIT B

John X1-8. 10/17/2000

improve this system

1 mutant + 1000

To select a non-interacting intein

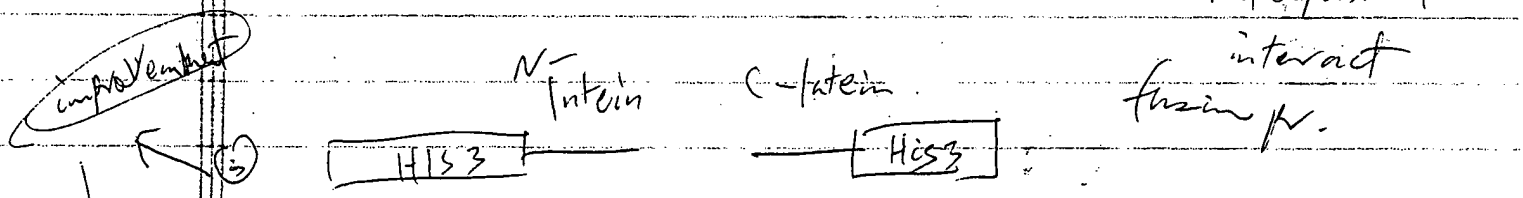
two constructs

	URA+	Ura-
FOA	-	+
Ura	+	-

pheS

10/17/2000 met C at 2:00 pm w/ Kirill Ostrovin & John Manfredi

Same construct to select inteins still capable of interact



fusion pr.

use 3-amino triazole Competitive inhibitor of the HIS3 enzyme

to reduce background

mutate → induce cleavage by T, pH, chemical reagents

pheS thermosensitive E. coli

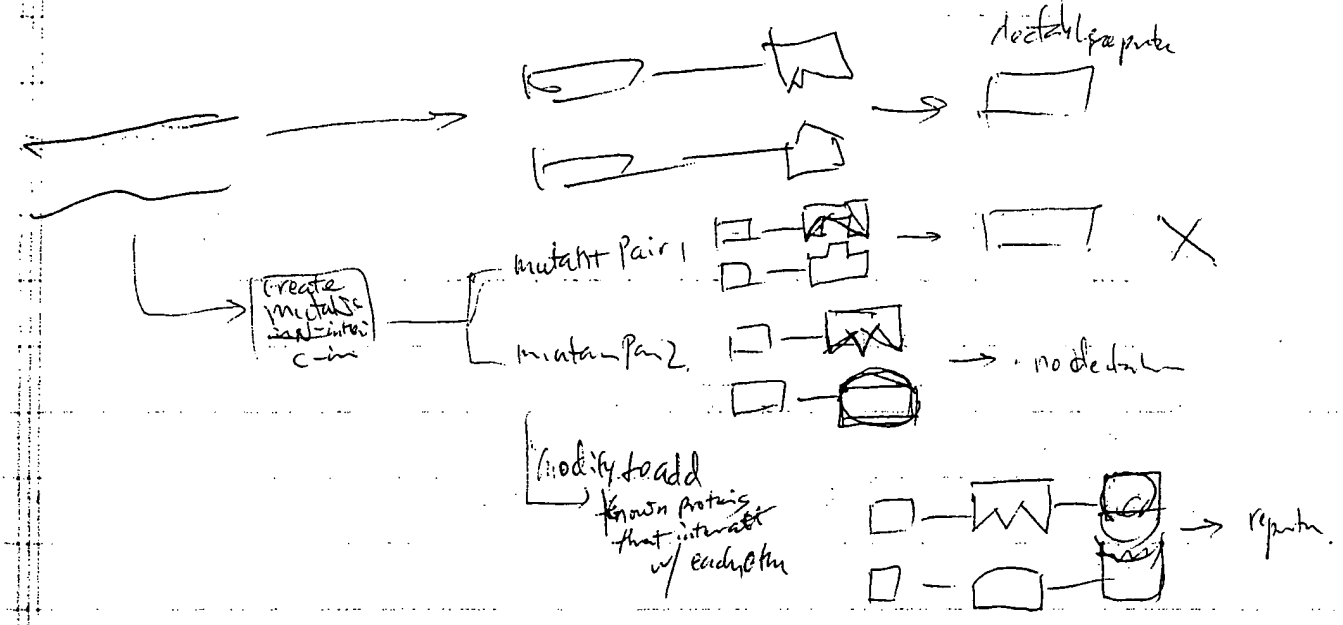
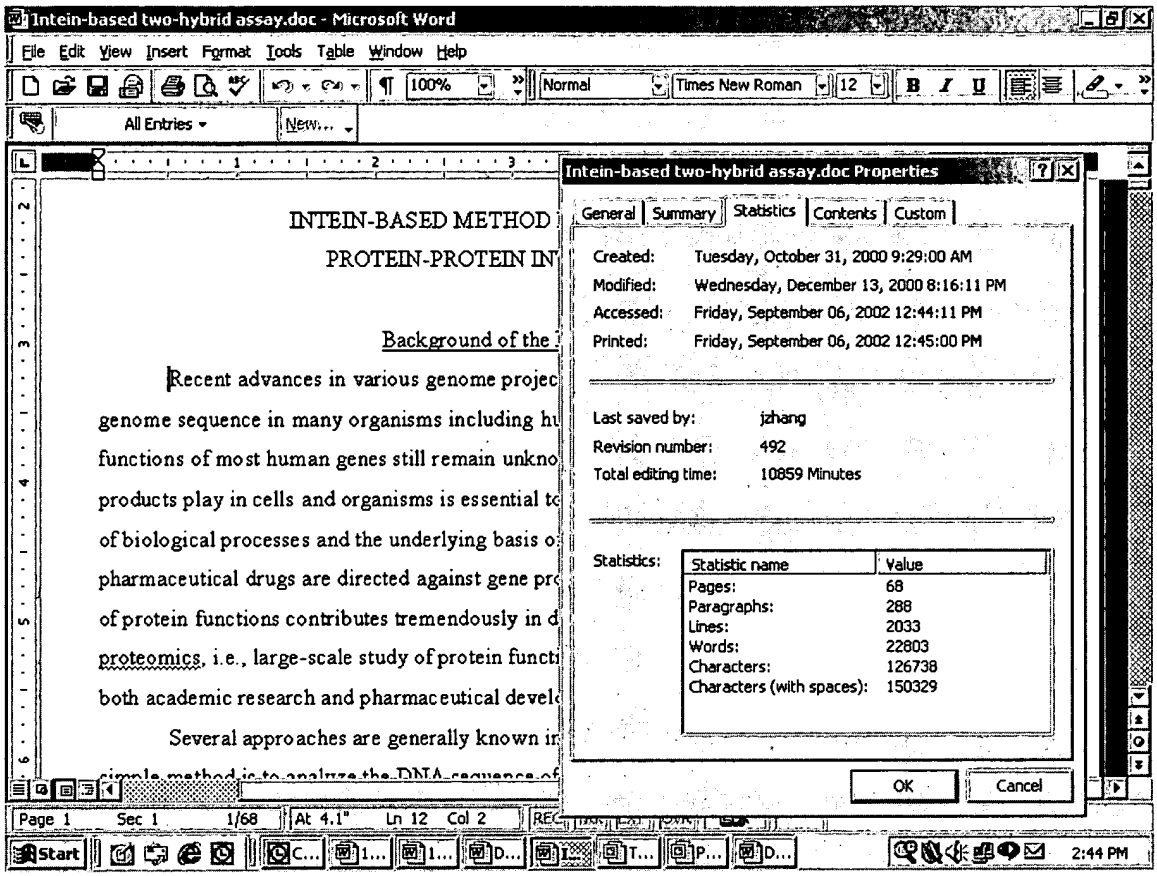

create mutant-interaction
mutant Pair 1
mutant Pair 2
modify to add
known protein that interacts w/ enzyme

EXHIBIT C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INTEIN-BASED METHOD
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Recent advances in various genome projec
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products play in cells and organisms is essential tc
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Several approaches are generally known ir
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EXHIBIT D

INTEIN-BASED METHOD FOR DETECTING PROTEIN-PROTEIN INTERACTIONS

Background of the Invention

Recent advances in various genome projects are resulting in the revelation of genome sequence in many organisms including humans. However, the biological functions of most human genes still remain unknown. The elucidation of the roles gene products play in cells and organisms is essential to the understanding of the mechanisms of biological processes and the underlying basis of diseases. In particular, most pharmaceutical drugs are directed against gene products, i.e., protein targets. Knowledge of protein functions contributes tremendously in drug development. Therefore, proteomics, i.e., large-scale study of protein functions is gaining increased attention in both academic research and pharmaceutical development.

Several approaches are generally known in the art for studying gene functions. A simple method is to analyze the DNA sequence of a particular gene and the amino acid sequence coded by the gene and compare the sequences to the sequences of those genes with known functions. Generally, similar functions can be predicted based on sequence homologies. This "homology method" has been widely used and powerful computer programs have been designed to facilitate homology analysis. *See, e.g.,* Altschul *et al., Nucleic Acids Res.,* 25:3389-3402 (1997). However, this method is useful only when the function of a homologous gene is known.

Another useful approach is to interfere with the expression of a particular gene in a cell or organism or simply delete the gene and examine the associated specific phenotypical changes. For example, Fire *et al., Nature,* 391:806-811 (1998) discloses a

"RNA interference" assay in which double-stranded RNA transcripts of a particular gene is injected into cells or organisms to determine the phenotypes caused by the exogenous RNA. Alternatively, transgenic technologies can be utilized to delete or "knock out" a particular gene in an organism and the effect of the gene knockout is determined. *See e.g.*, Winzeler *et al., Science,* 285:901-906 (1999); Zambrowicz *et al., Nature,* 392:608-611 (1998). The phenotypical effects resulting from the disruption of particular gene expression can shed some light on the functions of the gene. However, the techniques involved are complex and the required time for a phenotype to appear can be very long especially in animals. In addition, in many cases, disruption of a particular gene may not cause any detectable phenotypical effect.

Gene functions can also be uncovered by genetic linkage analysis, e.g., by positional cloning to identify the gene(s) responsible for certain diseases, or by comparing and analyzing the genetic variations among different individuals in a population and the various symptoms associated with the genetic variations. Linkage analysis is a powerful tool particularly when genetic variations exist in a traceable population from which samples are readily obtainable. However, readily identifiable genetic diseases are rare and samples from a large population with genetic variations are not easily accessible. In addition, it is also possible that a gene identified in a linkage analysis does not contribute to the associated disease or symptom but rather is simply linked to other unknown genetic variations that cause the phenotypic defects.

With the advance of bioinformatics and publication of the full genome sequence of many organisms, computational methods have also been developed to assign protein functions by comparative genome analysis. For example, Pellegrini *et al., Proc. Natl. Acad. Sci. USA* 96:4285-4288 (1999) discloses a phylogenetic profiling method in which a "phylogenetic profile" is established summarizing the pattern of presence or absence of a particular protein across a number of organisms as determined by analyzing the genome sequences of the organisms. A protein's function is predicted to be linked to another protein's function if the two proteins share the same phylogenetic profile. Another method, the Rosetta Stone method, is based on the theory that very often separate proteins in one organism are expressed as separate domains of a fusion protein in some other organisms. Because the separate domains in the fusion protein are predictably

associated with the same function, it can be reasonably predicted that the separate proteins are associated with same functions. Therefore, by discovering separate proteins corresponding to a fusion protein, i.e., the "Rosetta Stone sequence," functional linkage between proteins can be established. *See* Marcotte *et al., Science,* 285:751-753 (1999); Enright *et al., Nature,* 402:86-90 (1999). Another computational method is the "gene neighbor method." *See* Dandekar *et al., Trends Biochem. Sci.,* 23:324-328 (1998); Overbeek *et al., Proc. Natl. Acad. Sci. USA* 96:2896-2901 (1999). This method is based on the belief that if two genes are found neighbors in several different genomes, the proteins encoded by the genes are likely associated with a common function.

While the above-described methods are useful in analyzing protein functions, they are constrained by various practical limitations such as availability of suitable samples, inefficient assay procedures, and reliabilities. The computational methods are useful in linking proteins together by functions. However, they are only applicable to certain proteins, and the linkage maps established therewith are sketchy and lack concrete information on how proteins function in relation to each other within the functional network. Indeed, none of the above-described methods place the identified protein functions in the context of protein-protein interactions.

In contrast with the traditional view of protein function which focuses on the action of a single protein molecule, the modern expanded view of protein function defines a protein as an element in an interaction network. *See* Eisenberg *et al., Nature,* 405:823-826 (2000). That is, a full understanding of the function of a protein will require knowledge of not only the characteristics of the protein itself, but also its interactions or connections with other proteins in the same interacting network. In essence, protein-protein interactions form the basis of almost all biological processes, and each biological process or cell machine is composed of a network of interacting proteins. For example, cellular structures such as cytoskeletons, nuclear pores, centrosomes, and kinetochores are formed by complex interactions among a multitude of proteins. Many enzymatic reactions are associated with large protein complexes formed by interactions among enzymes, protein substrates and protein modulators. In addition, protein-protein interactions are also part of the mechanisms for signal transduction and other basic cellular functions such as DNA replication, transcription, and translation. For example,

the complex transcription initiation process generally requires protein-protein interactions among numerous transcription factors, RNA polymerase, and other proteins. *See e.g.*, Tjian and Maniatis, *Cell,* 77:5-8 (1994).

Because most proteins function through their interactions with other proteins, if a test protein interacts with a known protein, one can reasonably predict that the test protein is associated with the functions of the known protein, e.g., in the same cellular structure or same cellular process as the known protein. Thus, interaction partners can provide an immediate and reliable understanding towards the functions of the interacting proteins. By identifying interacting proteins, a better understanding of disease pathways and the cellular processes that result in diseases can be achieved, and important regulators and potential drug targets in disease pathways can be identified.

There has been much interest in protein-protein interactions in the field of proteomics. A number of biochemical approaches have been used to identify interacting proteins. These approaches generally employ the affinities between interacting proteins to isolate proteins in a bound state. Examples of such methods include coimmunoprecipitation and copurification optionally combined with cross-linking to stabilize the binding. Identities of the isolated protein interacting partners can be characterized by, e.g., mass spectrometry. *See e.g.*, Rout *et al., J. Cell. Biol.,* 148:635-651 (2000); Houry *et al., Nature,* 402:147-154 (1999); Winter *et al., Curr. Biol.,* 7:517-529 (1997). A popular approach useful in large-scale screening is the phage display method, in which filamentous bacteriophage particles are made by recombinant DNA technologies to express a peptide or protein of interest fused to a capsid or coat protein of the bacteriophage. A whole library of peptides or proteins of interest can be expressed and a bait protein can be used to screening the library to identify peptides or proteins capable of binding to the bait protein. *See e.g.*, U.S. Patent Nos. 5,223,409; 5,403,484; 5,571,698; and 5,837,500. However, the phage display method only identifies those proteins capable of interacting in an *in vitro* environment, while the coimmunoprecipitation and copurification methods are not amenable to high throughput screening.

The yeast two-hybrid system is a genetic method that overcomes the shortcomings of the above approaches. The yeast two-hybrid system has proven to be a

powerful method for the discovery of specific protein interactions *in vivo*. *See generally,* Bartel and Fields, eds., *The Yeast Two-Hybrid System*, Oxford University Press, New York, NY, 1997. The yeast two-hybrid technique is based on the fact that the DNA-binding domain and the transcriptional activation domain of a transcriptional activator contained in different fusion proteins can still activate gene transcription when they are brought into proximity to each other. As shown in Figure 1, in a yeast two-hybrid system, two fusion proteins are expressed in yeast cells. One has a DNA-binding domain of a transcriptional activator fused to a test protein. The other, on the other hand, includes a transcriptional activating domain of the transcriptional activator fused to another test protein. If the two test proteins interact with each other *in vivo*, the two domains of the transcriptional activator are brought together reconstituting the transcriptional activator and activating a reporter gene controlled by the transcriptional activator. *See, e.g.,* U.S. Patent No. 5,283,173.

Because of its simplicity, efficiency and reliability, the yeast two-hybrid system has gained tremendous popularity in many areas of research. Numerous protein-protein interactions have been identified using the yeast two-hybrid system. The identified proteins have contributed significantly to the understanding of many signal transduction pathways and other biological processes. For example, the yeast two-hybrid system has been successfully employed in identifying a large number of novel cell cycle regulators that are important in complex cell cycle regulations. Using known proteins that are important in cell cycle regulation as baits, other proteins involved in cell cycle control were identified by virtue of their ability to interact with the baits. *See generally,* Hannon *et al., in The Yeast Two-Hybrid System*, Bartel and Fields, eds., pages 183-196, Oxford University Press, New York, NY, 1997. Examples of cell cycle regulators identified by the yeast two-hybrid system include CDK4/CDK6 inhibitors (e.g., p16, p15, p18 and p19), Rb family members (e.g., p130), Rb phosphatase (e.g., PP1-α2), Rb-binding transcription factors (e.g., E2F-4 and E2F-5), General CDK inhibitors (e.g., p21 and p27), CAK cyclin (e.g., cyclin H), and CDK Thr161 phosphatase (e.g., KAP and CDI1). *See id.* "[T]he two-hybrid approach promises to be a useful tool in our ongoing quest for new pieces of the cell cycle puzzle." *See id* at page 193. In another example, the yeast two-hybrid system proved to be a powerful approach in analyzing the yeast pheromone

response pathway, a complex multistep signal transduction process in haploid yeast cell mating. *See generally,* Sprague *et al., in The Yeast Two-Hybrid System*, Bartel and Fields, eds., pages 173-182, Oxford University Press, New York, NY, 1997. As described in Sprague, various genes were isolated from mutant yeast strains having altered pheromone response patterns. However, it was not clear how the proteins encoded by these genes function in the pheromone response pathway. The yeast two-hybrid system was utilized to test such proteins and mutant forms thereof for their ability to interact with each other. As a result, new insights and better understandings of the complex process were achieved. *See id.*

The classic yeast two-hybrid system depends on gene activation in yeast nucleus and has generally required that specific protein-protein interactions between fusion proteins occur within the nucleus of yeast cells. Thus, although the conventional yeast two-hybrid system has been used successfully in the discovery of numerous protein interactions, its usefulness may be limited when it is used in detecting those protein-protein interactions that require non-nuclear environment. For example, many cell surface proteins and their ligands contain disulfide bonds, which can be disrupted under the intracellular reducing conditions. Additionally, posttranslational protein modifications, particularly glycosylation, typically would preclude the nuclear localization of the modified proteins.

Cytosolic and cell surface protein-protein interactions play major roles in normal cellular functions and biological responses. In particular, many cytosolic and cell surface protein-protein interactions are involved in disease pathways. For example, attacks by pathogens such as viruses and bacteria on mammalian cells typically begin with interactions between viral or bacterial proteins and mammalian cell surface proteins. Therefore, there is a need in the art for improved methods that can be used to efficiently detect cytosolic and cell surface protein-protein interactions.

Summary of the Invention

This invention provides a versatile and sensitive assay system for detecting protein-protein interactions that circumvents the above-described limitations inherent in prior art methods. Particularly, the present invention utilizes the so-called inteins, which

are peptide sequences capable of directing protein trans-splicing both *in vivo* and *in vitro*. An intein is an intervening protein sequence in a protein precursor that is excised off from the protein precursor during protein splicing. Protein splicing results in the concomitant ligation of the flanking protein fragments, i.e., the exteins, with a native peptide bond, thus forming a mature extein protein and the free intein. It is now known that inteins incorporated into non-native precursors can also cause protein-splicing and excision of the inteins. In addition, an N-terminal intein fragment in a fusion protein and a C-terminal intein fragment in another fusion protein, when brought into contact with each other, can bring about trans-splicing between the two fusion proteins. Thus, in accordance with the present invention, two hybrid fusion constructs are provided. One has a first test agent and an N-terminal intein fragment or N-intein, and the other has a second test agent and a C-terminal intein fragment or C-intein. In addition, one or both fusion constructs may have a reporter that undergoes detectable changes upon trans-splicing of the fusion constructs. If the first and second test agents interact with each other thus bringing the N-intein and C-intein to close proximity to each other, protein trans-splicing takes place. As a result, the fusion constructs are spliced and/or re-ligated causing detectable changes in the reporter. Thus, by detecting the changes in the reporter, interactions between two test agents can be determined.

Intein-based trans-splicing can take place *in vitro* in a cell free environment. Therefore, the assay system of the present invention can be used for convenient and speedy *in vitro* analysis of protein-protein interactions. Particularly, the system can be easily adapted to high-throughput screening procedures.

In addition, trans-splicing can also occur inside a host cell in many different cellular backgrounds and compartments. In particular, unlike the traditional two-hybrid systems, the interacting proteins need not be transported into cell nucleus. Thus, the system is useful in determining protein-protein interactions that require specific cellular environment. For example, the system can be employed to detect interactions between nuclear proteins, cytosolic proteins, and between membrane or extracellular proteins.

Additionally, protein trans-splicing mediated by the N-intein and C-intein is independent of other cellular factors and does not require the action of other proteins such as proteases and the like. This makes the assay system of the present invention more

reliable and easier to perform as compared to the assay methods known in the art for detecting protein-protein interactions.

Another distinct feature of the intein-based assay is that the detection of protein-protein interaction is based on the occurrence of protein trans-splicing events, which typically are associated with protein cleavage and result in new protein structures and functions. Thus, the numerous direct and indirect methods available in the art for detecting changes in protein structures and functions allow great flexibility in adapting the system to appropriate detection techniques and optimizing the system.

Accordingly, in accordance with a first aspect of the present invention, a method for detecting protein-protein interaction is provided. Briefly, two fusion proteins are prepared and allowed to interact with each other. One of the two fusion proteins includes an N-intein and a first test polypeptide, and the other fusion protein includes a C-intein and a second test polypeptide. One or both of the two fusion proteins have an inactive reporter capable of being converted to an active reporter upon trans-splicing through the N-intein and the C-intein. The change in the active reporter level is determined. An increase in the amount of the active reporter would indicate that the first and second test polypeptides interact with each other through, e.g., binding affinity, to result in the trans-splicing of the two fusion proteins mediated by the N-intein and the C-intein. Preferably, the N-intein and C-intein are not associated with each other and do not exhibit any significant binding affinity to each other. Nor do they associate or bind to the inactive reporter or test polypeptides in the fusion proteins.

In one embodiment, the inactive reporter can be a polypeptide linked to one of the fusion proteins, and is cleaved off into a free form from the fusion protein upon protein trans-splicing. The reporter polypeptide can be selected and the fusion proteins can be designed such that the bound form of the polypepitde is inactive while the free reporter released from the fusion protein is active, i.e., is detectable directly or indirectly.

In another embodiment, one of the two fusion proteins has a reporter polypeptide linked to the N-terminus of the N-intein. The other fusion protein includes another reporter polypeptide linked to the C-terminus of the C-intein. Upon trans-splicing between the two fusion proteins through the N- and C-inteins, the two inactive reporter

polypeptides are ligated together with a peptide bond forming an active reporter protein, which is detectable directly or indirectly.

The assay can be conducted *in vitro* in a substantially cell free environment by mixing purified form of the two fusion proteins together under conditions suitable for protein interactions and for protein trans-splicing. Alternatively, the fusion proteins can be recombinantly expressed separately in different host cells, and cell lysates or crude extracts prepared from the cells can be mixed to allow protein-protein interaction. The active reporter protein is then detected.

The assay can also be conducted *in vivo* by allowing the fusion proteins to interact within a host cell. Suitable cells include, but are not limited to, bacteria cells, yeast cells, plant cells, insect cells and animal cells. Chimeric genes encoding the above-described fusion proteins are introduced into a host cell to recombinantly express the fusion proteins. The presence or absence, or increase of the active reporter protein in the host cell is determined. In one embodiment, a first chimeric gene encoding one of the two fusion proteins is expressed in a haploid Saccharomyces cell of a mating type and a second chimeric gene encoding the other fusion protein is expressed in another haploid Saccharomyces cell of a different mating type. The two cells are mated to form a diploid Saccharomyces cell and any change in the amount of the active reporter protein in the diploid Sacharomyces cell is then determined.

In a specific embodiment of the *in vivo* assay, expression of one or more of the chimeric genes can be made inducible, e.g., by placing the genes under control of an inducible promoter, such that one or more of the fusion proteins are produced when the host cell is subject to a predetermined condition.

In yet another embodiment of the *in vivo* assay, the fusion proteins can have a signal peptide and optionally a membrane anchoring domain such that the fusion proteins recombinantly expressed in the host cells are secreted extracellularly or anchored on cell surface.

In addition, the assay can also be conducted in the presence of a third polypeptide. In this manner, the interaction between the first and second test polypeptides can be detected if the interaction requires the presence of the third polypeptide. The third polypeptide may be a protein having affinity to either the first or second test polypeptides

or both. Alternatively, the third polypeptide can modify one or both test polypeptides, e.g., by phosphorylation, glycosylation, and the like.

The techniques used for monitoring the occurrence of protein trans-splicing events and detecting an active reporter will depend on the inactive reporter used and the active reporter derived therefrom. The system of the present invention can be designed such that an active reporter can be detected based on changes in protein sizes or other properties, or activation of certain protein functions. For example, in an *in vivo* system, detection of an active reporter can be based on cell viability assays, color assays, and the like.

In accordance with a second aspect of the present invention, the above-described assay system is employed to determine whether a compound is capable of interfering with an interaction between a first polypeptide and a second polypeptide. Essentially, two fusion proteins as described above are provided except that the first and second polypeptides are known to interact with each other. The interaction between the two fusion proteins in the presence of the test compound is determined. Many suitable reporters can be used in this screening assay system. Preferably, a reverse or negative selection technique is incorporated into the screening assay of the present invention. For example, the fusion proteins can be designed such that the active reporter generated by protein trans-splicing is toxic to the host cell or represses the expression of a detectable gene. In this manner, compounds capable of interfering with the interaction between the two fusion proteins can be identified based on, e.g., the survival of the host cell or the expression of a detectable gene.

In accordance with another aspect of the present invention, a composition for detecting protein-protein interactions is provided, which includes a first chimeric gene encoding a first fusion protein and a second chimeric gene encoding a second fusion protein. The first fusion protein includes an N-intein and a first test polypeptide. The second fusion protein has a C-intein and a second test polypeptide. One or both of the fusion proteins has an inactive reporter protein capable of being converted to an active reporter protein upon trans-splicing through the N-intein and the C-intein. In one embodiment, each of the chimeric genes is contained in an expression vector, respectively. The expression vectors also contain elements necessary for the replication

of the vector in a host cell, the correct transcription and translation of the chimeric genes (e.g., promoters and other transcriptional regulatory elements, transcription termination signal, etc.). The vectors preferably also contain a selection marker gene for selecting those host cells harboring the expression vectors . Preferably, the N-intein and the C-intein are incapable of interacting with each other or with the inactive reporter.

The foregoing and other advantages and features of the invention, and the manner in which the same are accomplished, will become more readily apparent upon consideration of the following detailed description of the invention taken in conjunction with the accompanying examples and drawings, which illustrate preferred or exemplary embodiments.

Brief Description of the Drawings

Figure 1 is an illustration of the classic yeast two-hybrid system known in the art;

Figure 2A illustrates a genetic selection process for selecting N-inteins and C-inteins that do not interact with each other;

Figure 2B shows a process for verifying that the selected non-interacting N-intein and C-intein are capable of mediating protein trans-splicing;

Figures 3A-3F are diagrams illustrating the fusion constructs in different embodiments of the present invention;

Figure 4 is a drawing demonstrating the use of the protein encoded by the *URA3* gene as a reporter protein in one embodiment of the present invention;

Figure 5 shows an embodiment of the present invention in which a transcriptional activator is used as an active reporter which drives the expression of the selection marker gene *URA3*;

Figure 6 is a diagram illustrating an embodiment of the present invention in which a modifying enzyme is expressed in a multi-hybrid system and interaction between the modified proteins is detected;

Figure 7 is an illustration of an embodiment of the present invention in which the intein-based hybrid system is used to detect an interaction between extracellular proteins.

Detailed Description of the Invention

As used herein, the terms "polypeptide," "protein," and "peptide" are used interchangeably to refer to amino acid chains in which the amino acid residues are linked by covalent peptide bonds. The amino acid chains can be of any length of at least two amino acids, including full-length proteins. Unless otherwise specified, the terms "polypeptide," "protein," and "peptide" also encompass various modified forms thereof, including but not limited to glycosylated forms, phosphorylated forms, etc.

The term "test agent" means a chemical compound, preferably an organic compound, to be tested in the present invention to determine its ability to interact with another chemical compound. Test agents may include various forms of organic compounds, or combinations or conjugates thereof. In one embodiment, the test agents preferably are polypeptides, in which case the test agents are termed "test polypeptides" or "test proteins."

The term "fusion construct" refers to a non-naturally occurring hybrid or chimeric construct having two or more distinct portions covalently linked together, each portion being or being derived from a specific molecule. When two or more portions in a fusion construct as defined above are polypeptides and are linked together by peptide bonds, the fusion construct is conveniently referred to as "fusion protein."

As used herein, the term "interacting" or "interaction" means that two domains or independent entities exhibit sufficient physical affinity to each other or form chemical bonds between them so as to bring the two "interacting" domains or entities physically close to each other. Examples of physical affinities and chemical bonds include but are not limited to, forces caused by electrical charge differences, hydrophobicity, hydrogen bonds, van der Wals force, ionic force, covalent linkages, and combinations thereof. The state of proximity between the interacting domains or entities may be transient or permanent, reversible or irreversible. In any event, it is in contrast to and distinguishable from contact caused by natural random movement of two entities. Typically although not necessarily, an "interaction" is exhibited by the binding between the interacting domains or entities. Examples of interactions include specific interactions between antigen and antibody, ligand and receptor, and the like. **[PLEASE VERIFY. IS THERE ANY**

BETTER WAY, MAY BE QUANTATITIVE METHOD, TO DEFINE THE TERM?]

As used in the present disclosure, the term "reporter" means a molecule or a moiety or domain thereof that can be used as a marker for the determination of the occurrence of protein trans-splicing. An "inactive reporter" is a form of the reporter that is not detectable by a particular detection means, while an "active reporter" is a form of the reporter that is detectable by that detection means. It should be recognized that the terms "detectable" and "not detectable" are used herein in a relative sense. In essence, there should be a measurable or detectable change in the reporter, either quantitative or qualitative, upon intein-based trans-splicing. For purposes of the present discussion, "active reporters" include both reporters that are directly detectable and those reporters that are detectable indirectly. One example of an indirectly detectable active reporter is a transcription activator that can activate the transcription of a detectable gene and thus cause the synthesis of a detectable protein encoded by the detectable gene.

Many reporters are known in the art and the selection and application of any of those reporters to the present invention should be apparent to a skilled artisan apprised of the present disclosure. Examples of reporters suitable for use in a yeast system or other systems include, but are not limited to, β-galactosidase (β-Gal) encoded by the *LacZ* gene which converts white X-Gal into a product with a blue color, product of the CYH^S which confers sensitivity to cycloheximide (CYH), proteins encoded by the auxotrophic genes *URA3, HIS3, LEU2,* and *TRP1*, and green fluorescent protein (GFP) which can be sorted by flow-activated cell sorting (FACS). *See* Cubitt *et al., Trends Biochem. Sci.*, 20:448-455 (1995).

Typically, an inactive reporter can be converted to an active reporter upon trans-splicing in the method of this invention. For example, a molecule when fused to a fusion construct of the present invention may not be detectable and thus is referred to as "an inactive reporter." The fused form may be released from the fusion construct into a free form of the molecule that is detectable. This detectable free form is referred to as an "active reporter," which is in contrast to the "inactive" undetectable bound form of the reporter. In another example, two inactive reporters are fused to an N-intein and a C-intein, respectively, and upon trans-splicing, the two inactive reporters are ligated

together forming a detectable active reporter. For this purpose, fragments of an active reporter that are not detectable can also be referred to "inactive reporter." Thus, an N-terminal fragment of a reporter protein is fused to an N-intein and a C-terminal fragment of the reporter protein is fused to a C-intein. Upon protein trans-splicing mediated by the N- and C-intein, the N-terminal and C-terminal fragments can be religated in-frame forming a full-length detectable active reporter protein.

As is known in art, inteins are intervening protein sequences in protein precursors which are exercised out, or removed, from the protein precursors during protein splicing. The protein sequences flanking inteins are called exteins. The excision of an intein is associated with the concomitant ligation of the N-extein (the protein sequence to the N-terminus of the intein) and the C-extein (the protein sequence to the C-terminus of the intein) through a native peptide bond thus forming a mature extein protein and a free intein. *See* Perler *et al., Nucleic Acids Res.,* 22:1125-1127 (1994). The entire protein splicing process is autocatalyzed by the intein and is believed to be independent of specific host cell factors. Indeed, intein-based protein splicing has been shown to occur *in vitro* as well as in heterologous organisms. *See* Perler *et al., Cell,* 92:1-4 (1998). Intein-based protein splicing has also been shown to be independent of the native flanking exteins. Hybrid protein sequences containing inteins fused to non-native polypetide sequences are able to undergo protein splicing to excise the inteins and ligate the flanking polypeptide sequences. *See e.g.,* Evans *et al., J. Biol. Chem.,* 274:3923-3926 (1999); Evans *et al., J. Biol. Chem.,* 275:9091-9094 (2000).

Certain amino acid sequences within an intein sequence are irrelevant to protein splicing. Based on sequence comparison and structural analysis, it is now known that the residues responsible for splicing are the intein N-terminal about 100 amino acids and the intein C-terminal about 50 amino acids. *See e.g.,* Duan *et al., Cell,* 89:555-564(1997), Hall *et al., Cell,* 91:85-97 (1997); Klabunde *et al., Nature Struct. Biol.* 5:31-36 (1998). Indeed, a functional mini-intein can be produced by deleting the centrally located irrelevant amino acid sequence leaving the N-terminal sequence of about 100 amino acids fused directly to the C-terminal sequence of about 50 amino acids. *See e.g.,* Wu *et al., Biochim. Biophys. Acta.,* 1387:422-32 (1998). In addition, it has been discovered that even when the N-terminal intein sequence and the C-terminal intein sequence are in

different proteins, they are still able to mediate protein trans-splicing. *See id.; see also,* Shingledecker *et al., Gene,* 207:187-195 (1998); Evans *et al., J. Biol. Chem.*, 274:3923-3926 (1999); Evans *et al., J. Biol. Chem.*, 275:9091-9094 (2000).

The present invention utilizes the trans-splicing capability of inteins to provide a method for detecting interactions between test agents such proteins. Thus, in accordance with the present invention, two fusion constructs are provided: one has a first test agent and an N-intein, and the other has a second test agent and a C-intein. In addition, one or both fusion constructs have a reporter that undergoes detectable changes upon intein-mediated trans-splicing of the fusion constructs. If the first and second test agents interact with each other and bring the N-intein and C-intein into close proximity to each other, protein trans-splicing takes place. As a result, the fusion constructs are trans-spliced and/or re-ligated causing detectable changes in the reporter. By detecting the changes in the reporter, the interaction between two test agents can be determined.

As used herein, the terms "N-intein" and "C-intein" refer to an N-terminal and a C-terminal portion of an intein, respectively. An N-intein itself alone cannot direct protein splicing, and likewise, a C-intein itself alone is incapable of catalyzing protein splicing. However, when an N-intein and a C-intein are placed in close proximity, they are capable of acting in concert to catalyze protein trans-splicing. Conserved intein motifs have been identified in many inteins. Typically, an intein includes an N-terminal splicing region having the motifs of blocks A, N_2, B, and N_4, an endonuclease or linker domain region having the motifs of blocks C, D, E, and H, and a C-terminal splicing region having the motifs of blocks F and G. *See* Pietrokovski, *Protein Sci.,* 3:2340-2350 (1994); Pietrokovski, *Protein Sci.,* 7:64-71 (1998). Thus, in a specific embodiment, N-intein includes at least the motifs of blocks A, N_2, B, and N_4, while C-intein includes at least the motifs of blocks F and G. Typically, "N-intein" is an amino acid sequence matching the N-terminal sequence of about 90 to 110 amino acids of an intein, while "C-intein" is an amino acid sequence matching the C-terminal sequence of about 30 to 50 amino acids of an intein. **[PLEASE VERIFY.]** A skilled artisan will recognize that optimal sequences of N-inteins and C-inteins can be determined by routine trial and error experiments. In addition, it should understood that the terms "N-intein" and "C-intein" also encompass non-native or modified amino acid sequences that are derived from an N-

terminal or C-terminal portion of an intein, respectively, e.g., modified or mutein forms containing amino acid insertions, deletions, or substitutions.

Protein precursors containing inteins have been found in all three life domains: archaea, bacteria, and eucarya. A large number of inteins exist in bacteria and yeast. *See* Perler *et al., Nucleic Acids Res.*, 28:1 344-5 (2000); *see also InBase, the New England Intein Database, at* http://www.neb.com/neb/inteins.html. The N-intein and C-intein used in the fusion constructs of the present invention can be selected according to the naturally occurring intein sequences. Alternatively, the naturally occurring intein sequences can be modified by deleting, inserting, or substituting amino acids to generate desirable properties in the N- and C-intein.

Some naturally occurring native N-inteins and C-inteins are known to interact with each other. This may cause certain background noises and false positives. To minimize the background and increase the assay sensitivity in the present invention, it is preferred to use an N-intein and a C-intein that do not substantially interact with each other. That is, they do not exhibit sufficient physical affinity to each other or form chemical bonds between them so as to bring them physically close to each other to cause substantial protein trans-splicing. **[PLEASE VERIFY. IS THERE ANY BETTER WAY, MAY BE QUANTATITIVE METHOD, TO DEFINE NON-INTERACTION?]**

If the N-intein and C-intein have relatively high affinity to each other, the N-inten and C-intein can be mutated to minimize their interaction. Alternatively, as will be described in detail below, competitive inhibitors of the reporters can be applied to minimize background detection signals. In this way, the detection signal from the active reporter produced by the interaction between the test proteins will be sufficiently greater than the background detection signal such that the interaction between the test proteins can be distinguished from the background interaction between the N-intein and C-intein.

Various trans-splicing assays may be used in combination with recombinant mutagenesis techniques to generate an N-intein and a C-intein that do not interact with each other and yet are capable of catalyzing protein trans-splicing when brought to proximity to each other. Conveniently, a genetic selection assay can be employed. For example, as shown in Figure 2A, two chimeric genes can be prepared using standard

recombinant DNA technologies. One chimeric gene encodes a fusion protein containing the N-terminal fragment of a reporter protein fused, at its C-terminus, to the N-terminus of an N-intein. The other chimeric gene encodes a fusion protein having a C-intein fused, at its C-terminus, to the N-terminus of the C-terminal fragment of a reporter protein. The N- and C-terminal fragments of the reporter protein should not interact with each other or with N- or C- intein. They can be in any length so long as an active reporter protein can be generated when they are ligated together through protein trans-splicing mediated by the N- and C-intein. The genetic selection assay can be performed in any suitable host cells, preferably conducted in the same type of cells in which the protein-protein interaction detection assay is conducted. The two chimeric genes are introduced to a host cell for the expression of the two fusion proteins. Alternatively, in the case yeast cells, they can be introduced into two yeast cells having different mating types, which are subsequently mated. If the N-intein and C-intein thus expressed interact with each other, an active reporter will be detectable in the host cell. To obtain N-inteins and C-inteins that do not interact with each other, the DNA coding regions for the N-intein and C-intein are mutated using standard mutagenesis techniques to create changes in the amino acid sequences of the N- and C-intein. The thus generated mutant chimeric genes are then introduced into host cells for the genetic selection assay described above. In this manner, random mutations can be caused in the N- and C-intein and those mutant N-inteins and C-inteins that do not interact with each other are selected. *See* Figure 2A.

Besides random mutagenesis, site-directed mutagenesis can also be used to change amino acid sequences in wild-type N- and C-inteins in predetermined manners. For example, amino acid sequences can be modified to create consensus sequences for phosphorylation by protein kinases or for glycosylation. Alternatively, certain amino acids in wild-type N- and C-intein sequences can also be chemically modified, e.g., by incorporating non-natural amino acids or by chemically linking certain moieties to amino acid side chains.

The selection of non-interacting N-intein and C-intein can also be done in an *in vitro* assay. For example, fusion proteins containing wild-type or mutated N- or C-inteins expressed from the above-described chimeric genes can be purified by standard chromatographic or affinity techniques or prepared in crude cell extracts. Fusion protein

pairs (in which one contains an N-intein and the other contains a C-intein) are then mixed and incubated together *in vitro* under appropriate conditions to promote protein splicing as described below.

The thus selected N- and C-inteins are further tested for their ability to catalyze protein trans-splicing in a host cell. For this purpose, the selected chimeric genes containing desirable N- and C-intein coding sequences are further modified. Figure 2B illustrates an example of this verification process. Essentially, a pair of new chimeric genes are constructed and introduced into a host cell for expressing a pair of fusion proteins. One chimeric gene encodes a fusion protein containing the above-described N-terminal fragment of a reporter protein fused, at its C-terminus, to the N-terminus of an N-intein, and a bait protein fused to the C-terminus of the N-intein. The other chimeric gene encodes a fusion protein having a C-intein fused, at its C-terminus, to the N-terminus of the above-described C-terminal fragment of a reporter protein, and a prey protein fused to the N-terminus of the C-intein. The bait protein and prey protein are known to interact with each other. Any pair of interacting proteins known in the art can be used for this purpose. **[JOHN – CAN YOU SUPPLY SOME EXAMPLES?]** After the new chimeric genes are expressed in a host cell to produce the fusion proteins, the active reporter is detected to determine whether trans-splicing has occurred. In this manner, N-inteins and C-inteins that do not interact with each other but are nevertheless capable of mediating protein trans-splicing when they are brought into proximity can be identified.

It should be recognized that, although much of the description below is focused on protein-protein interactions, the method of the present invention for detecting interactions is applicable to any test agents, preferably macromolecules. For example, interactions among macromolecules such as oligosaccharides, lipids, nucleic acids, proteins, organic molecules including steroids and other drugs, viruses, and cells can all be detected by the present method. Thus, in accordance with present invention, two fusion constructs can be provided, one having an N-intein and a first test agent and the other having a C-intein and a second test agent. At least one of the two fusion constructs has an inactive reporter capable of being converted to an active reporter upon trans-splicing mediated by the N-intein and the C-intein. The two fusion constructs are then

mixed and incubated together or allowed to contact with each other in other manners under appropriate conditions. Each of the two fusion constructs should be designed such that the interaction between the first and second test agents is determinable by detecting or measuring the active reporter in the assay system.

Optionally, a control assay is conducted in parallel to the detection assay. Typically, in the control assay, the potential interaction between the two test agents being assayed in the detection assay of this invention is pre-empted, eliminated or inhibited. For example, in one control assay, control fusion constructs are used, in which two known agents that do not interact with other are included in lieu of the first and second test agents, respectively. Because the known agents in the control fusion constructs do not interact with each other, any active reporter signal in the control assay is a background signal. Alternatively, in another control assay, the control fusion constructs do not contain the first or second test agents. In other words, the control fusion constructs are different from those in a detection assay in that the control fusion constructs do not contain test agents. Thus, any active reporter signal in the control assay would not be the result of interaction between the test agents.

Preferably, a control assay utilizes the same two fusion constructs as those in a detection assay, which contain a first and a second test agent, respectively. However, the control assay is conducted in the presence of an inhibitor that interferes with the interaction between the first and second test agents in the fusion constructs. Typically, the inhibitor is an agent that interacts with one of the two test agents in a manner such that the interaction between the two test agents is disrupted, and as a result, the active reporter that would normally be formed upon interaction between the two test agents is not produced. Conveniently, one of the two test agents is used as an inhibitor. Such an agent should be in a free unbound form or in a hybrid form that will not cause the formation of the active reporter upon an interaction between this hybrid form and the other test agent in one of the two fusion constructs. For example, if the test agent used as an inhibitor is a protein, it can be conveniently expressed from an expression vector containing a gene sequence encoding the protein. **[JOHN – CAN YOU THINK OF OTHER CONTROL ASSAYS? Given the trans-splicing papers by NEB, it may be important to come up with as many examples of control assays as possible. The**

NEB papers do not disclose control assays as they are not concerned with protein-protein interactions.]

The level of detectable active reporter in the control assay is compared to that in the detection assay. As a result, positive signals indicating specific interactions in the detection assay can be confirmed and distinguished from background signals inherent in the assay system. A control assay is especially useful when the N-intein and C-intein used in the fusion constructs can interact with each other.

Alternatively, measures can be taken to reduce background signals. For example, in the case when cells of a His^- yeast strain are used as host cells and the *HIS3* gene product (imidazole glycerol phosphate dehydratase) is used as a reporter, the compound 3-amino-1,2,4-triazole (3-AT) can be added to the medium on which the yeast cells in the assay are grown. 3-aminotriazole (3-AT) specifically inhibits the *HIS3*-encoded enzyme imidazole glycerol phosphate dehydratase which is required in yeast for the synthesis of the amino acid histidine. *See* Kishore *et al., Ann. Rev. Biochem.,* 57:627-663 (1988). As a result, a strong signal is required to confirm actual interaction between the test proteins. *See* Durfee *et al., Genes Dev.*, 7:555-569 (1993). Other examples of competitive inhibitors useful in minimizing background signals include 5-fluoroorotic acid (5-FOA, inhibitor of the *URA3* gene product), β-aminoadipate (inhibitor of the *LYS2* gene product), canavanine (inhibitor of the *CAN1* gene product), cycloheximide (inhibitor of the *CYH2* gene product), and the like.

As will be apparent to a skilled artisan, any arrangements of the components in the fusion constructs of the present invention can be adopted so long as the protein trans-splicing mediated by the N- and C-intein and initiated by a specific interaction between the test agents can be detected by measuring the active reporter produced during the protein splicing process.

In one embodiment, as shown in Figure 3A, one fusion construct has a first test agent X fused or conjugated to the C-terminus of an N-intein, while the other fusion construct has a second test agent Y fused to the N-terminus of a C-intein and a reporter R (inactive) fused to the C-terminus of the C-intein. Upon tans-splicing, the reporter is excised off and becomes a free detectable active reporter R^*.

In another embodiment, as shown in Figure 3B, one fusion construct has a first test agent X fused to the C-terminus of an N-intein and a reporter R (inactive) fused to the N-terminus of the N-intein. The other fusion construct includes a second test agent Y fused to the N-terminus of a C-intein. After trans-splicing mediated by the N- and C-intein, a detectable free active reporter R^* is released.

Figure 3C illustrates the fusion construct arrangement in another embodiment of the invention. The first fusion construct consists of a first portion of a reporter R (R_1) fused to the N-terminus of an N-intein and a first test agent (X) fused to the C-terminus of the N-intein. The second fusion construct consists of a second test agent (Y) fused to the N-terminus of a C-intein and the remaining portion of the reporter R (R_2) fused to the C-terminus of the C-intein. In this manner, upon intein-directed trans-splicing, the two portions of the reporter R are ligated together thus forming a detectable active reporter R.

Figure 3D is a diagram showing the fusion constructs design in yet another embodiment of the present invention. The first fusion construct consists of a first test agent (X) fused to a first portion of a reporter R (R_1) which in turn is fused to the N-terminus of an N-intein. The second fusion construct consists of a C-intein, the remaining portion of the reporter R (R_2) fused to the C-terminus of a C-intein, and a second test agent (Y) fused to R_2. If the test agents X and Y interact with each other to bring the N-intein and C-intein close together, trans-splicing will result in a detectable construct X-R-Y.

Yet another arrangement of the fusion constructs is demonstrated in Figure 3E. The first construct is composed of a first portion of a reporter R (R_1) fused to the N-terminus of an N-intein and a test agent (X) fused to the C-terminus of the N-intein. The second construct has a C-intein, the remaining portion the reporter R (R_2) fused to the C-terminus of the C-intein, and another test agent (Y) fused to R_2. Assuming test agents X and Y interact with each other thus bringing the N-intein and C-intein close together, trans-splicing can occur resulting in a detectable construct R-Y.

Figure 3F illustrates yet another possible arrangement of the fusion constructs in the present invention. As shown in Figure 3F, the first fusion construct has a test agent (X) fused to a first portion of a reporter R (R_1) which is in turn fused to the N-terminus of an N-intein. The second fusion construct includes another test agent (Y) fused to the N-

terminus of a C-intein and the remaining portion of the reporter R (R_2) fused to the C-terminus of the C-intein. Assuming test agents X and Y interact with each other thus bringing the N-intein and C-intein close together, trans-splicing can occur resulting in a detectable construct X-R.

As discussed above, the test agents can be any chemical compounds and are not limited to proteins. Likewise, both the inactive and active reporter(s) incorporated into the fusion constructs can be any suitable chemical compounds so long as specific and detectable changes can occur in the inactive reporter(s) during trans-splicing. The fusion constructs can be prepared by chemical synthesis and/or standard recombinant DNA techniques. For example, when the reporters or test agents are not protein, the N-intein and C-intein can be prepared by chemical synthesis or recombinant expression, and thereafter, the non-proteinaceous reporter or test agents can be chemically conjugated to the N-intein and/or C-intein through direct linkage or using a linker molecule. Methods for conjugating a protein or peptide to a molecule such as glycosaccharides, lipids, steroids, drugs, nucleic acids, and the like are known in the art and should be apparent to a skilled artisan apprised of the present disclosure. If both the test agents and reporters are proteins, the fusion constructs can be conveniently produced as fusion proteins by recombinantly expressing suitable chimeric genes. The fusion proteins can be extracted in a crude cell extract form or purified for *in vitro* assay. Purification can be achieved by conventional purification methods such as standard chromatographic or affinity techniques. Alternatively, for *in vivo* assays, the fusion proteins are expressed in suitable host cells and allowed to interact with each other within the host cells.

Although it is generally believed that intein-based protein splicing is independent of exteins, some studies have shown that the first amino acid in C-extein following a C-intein may be required unless the splicing reaction takes place in a reducing environment in the presence of a reducing thiol acid such as cysteine, mercaptoacetic acid, dithiothreitol, thiophenol, and the like. *See e.g.,* Paulus, *Annu. Rev. Biochem.*, 69:447-496 (2000); Severinov and Muir, *J. Biol. Chem.*, 273:16205-16209 (1998). Thus; when a non-protein inactive reporter or test agent is linked to the C-terminus of the C-intein in a fusion construct of the present invention, it is preferred that the non-protein entity is conjugated to the C-intein through a linker such as amino acid cystenine, serine, and

threonine. In the case of a polypeptide reporter or polypeptide test agent fused to the C-terminus of the C-intein, it may also be preferred that the first amino acid of the polypeptide immediately following the C-terminus of the C-intein is cystenine, serine, or threonine. In the event that the C-terminus of the C-intein is exposed and not fused to any moiety, it may be desirable to design the C-intein such that it includes an additional amino acid selected from cystenine, serine, and threonine. Alternatively, a reducing thiol acid such as cysteine, mercaptoacetic acid, dithiothreitol, thiphenol, and the like may be added to the assay system. In addition, where the N-terminus of an N-intein in the fusion constructs is linked to another non-protein moiety, it is also preferable that the chemical linkage between the N-intein and the non-protein moiety is an amide linkage and preferably a peptide bond. This can be achieved by using an amino acid as a linker linking the non-protein moiety to the N-terminus of the N-intein. **[JOHN, PLEASE VERIFY THIS PARAGRAPH.]**

The detection assay in accordance with the present invention can be conducted either *in vitro* or *in vivo* in a host cell. In an *in vitro* assay, the fusion constructs in crude cell extracts or in purified forms can be mixed and incubated together under appropriate conditions that promote interactions between the test agents. Methods for performing *in vitro* trans-splicing assays are disclosed, e.g., in U.S. Patent No. 5,834,247, which is incorporated herein by reference. It is noted that different agents may require different conditions for their interactions. As a starting point, for example, a buffer having 20 mM Tris-HCl, pH 7.0 and 500 mM NaCl may be used. Several different parameters may be varied, including temperature, pH, salt concentration, reducing agent, time, and the like. Some minor degree of experimentation may be required to determine the optimum incubation condition, this being well within the capability of one skilled in the art once apprised of the present disclosure. Cell free *in vitro* assays are especially suitable where the fusion constructs contain non-protein elements that cannot be synthesized by recombinant DNA technologies. In addition, *in vitro* assays also eliminate the constraints created by cell compartments and are useful in detecting interactions that may not be detectable in certain *in vivo* assays known in the art.

More conveniently, *in vivo* genetic assays are used in the detection method of the present invention. In this respect, fusion constructs, which normally are fusion proteins,

can be recombinantly expressed in a host cell by introducing into the host cell chimeric genes encoding the fusion proteins. For this purpose, the expression vectors and host cell strains used in various two-hybrid systems developed in the art may be adapted and incorporated in the assays. Such two-hybrid systems are generally disclosed in U.S. Patent Nos. 5,283,173; 5,525,490; 5,585,245; 5,637,463; 5,695,941; 5,733,726; 5,776,689; 5,885,779; 5,905,025; 6,037,136; 6,057,101; 6,114,111; and Bartel and Fields, eds., *The Yeast Two-Hybrid System*, Oxford University Press, New York, NY, 1997, all of which are incorporated herein by reference.

Typically, two chimeric genes are prepared encoding two fusion constructs as described above containing an N-intein and a C-intein, respectively. For the purpose of convenience, the two test polypeptides whose interaction is to be determined are referred to as "bait polypeptide" and "prey polypeptide," respectively. The chimeric genes encoding the fusion constructs containing the bait and prey polypeptides are termed "bait chimeric gene" and "prey chimeric gene," respectively. Typically, a "bait vector" and a "prey vector" are provided for the expression of a bait chimeric gene and a prey chimeric gene, respectively.

Many types of vectors can be used for the present invention. Methods for the construction of bait vectors and prey vectors should be apparent to skilled artisans in the art apprised of the present disclosure. *See generally, Current Protocols in Molecular Biology*, Vol. 2, Ed. Ausubel, *et al.*, Greene Publish. Assoc. & Wiley Interscience, Ch. 13, 1988; Glover, *DNA Cloning*, Vol. II, IRL Press, Wash., D.C., Ch. 3, 1986; Bitter, *et al.*, in *Methods in Enzymology* 153:516-544 (1987); *The Molecular Biology of the Yeast Saccharomyces*, Eds. Strathern *et al.*, Cold Spring Harbor Press, Vols. I and II, 1982; and Rothstein in *DNA Cloning: A Practical Approach*, Vol. 11, Ed. DM Glover, IRL Press, Wash., D.C., 1986.

Generally, the bait and prey vectors may include a promoter operably linked to a chimeric gene for the transcription of the chimeric gene, an origin of DNA replication for the replication of the vectors in host cells and a replication origin for the amplification of the vectors in, e.g., *E. coli*, and selection marker(s) for selecting and maintaining only those host cells harboring the vectors. Additionally, the vectors preferably also contain inducible elements, which function to control the expression of the chimeric gene.

Making the expression of the chimeric genes inducible and controllable is especially important in the event that the fusion proteins or components thereof are toxic to the host cells. Other regulatory sequences such as transcriptional termination sequences and translation regulation sequences (e.g., Shine-Dalgarno sequence) can also be included. An epitope tag coding sequence for detection and/or purification of the fusion proteins can also be incorporated into the expression vectors. Examples of useful epitope tags include, but are not limited to, influenza virus hemagglutinin (HA), Simian Virus 5 (V5), polyhistidine (6xHis), *c-myc*, lacZ, GST, and the like. Proteins with polyhistidine tags can be easily detected and/or purified with Ni affinity columns, while specific antibodies to many epitope tags are generally commercially available. The vectors can be introduced into the host cells by any techniques known in the art, e.g., by direct DNA transformation, microinjection, electroporation, viral infection, lipofection, gene gun, and the like. The bait and prey vectors can be maintained in host cells in an extrachromosomal state, i.e., as self-replicating plasmids or viruses. Alternatively, one or both vectors can be integrated into chromosomes of the host cells by conventional techniques such as selection of stable cell lines or site-specific recombination.

The *in vivo* assays of the present invention can be conducted in many different host cells, including but not limited to bacteria, yeast cells, plant cells, insect cells, and mammalian cells. A skilled artisan will recognize that the designs of the vectors can vary with the host cells used. In one embodiment, the assay is conducted in prokaryotic cells such as *Escherichia coli*, Salmonella, Klebsiella, Pseudomonas, Caulobacter, and Rhizobium. Suitable origins of replication for the expression vectors useful in this embodiment of the present invention include, e.g., the ColE1, pSC101, SV40 and M13 origins of replication. Examples of suitable promoters include, for example, the Gal10 promoter, the lacZ promoter, and the like. In addition, inducible promoters are also useful in modulating the expression of the chimeric genes. For example, the lac operon from bacteriophage lambda plac5 is well known in the art and is inducible by the addition of IPTG to the growth medium. Other known inducible promoters useful in a bacteria expression system include pL of bacteriophage λ, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like. Termination sequences such as the bovine growth hormone, SV40, lacZ and AcMNPV polyhedral polyadenylation signals may also be operably

linked to the chimeric gene. Other regulatory sequences such as leader sequences, enhancers, signal peptide sequences, membrane localization sequences can also optionally be included. **[PLEASE VERIFY.]**

In addition, selection markers sequences for selecting and maintaining only those prokaryotic cells expressing the desirable fusion proteins should also be incorporated into the expression vectors. Numerous selection markers including auxotrophic markers and antibiotic resistance markers are known in the art and can all be useful for purposes of this invention. For example, the *bla* gene which confers ampicillin resistance is the most commonly used selection marker in prokaryotic expression vectors. Other suitable markers include genes that confer neomycin, kanamycin, or hygromycin resistance to the host cells. In fact, many vectors are commercially available from vendors such as Invitrogen Corp. of San Diego, Calif., Clontech Corp. of Palo Alto, Calif., BRL of Bethesda, Maryland, and Promega Corp. of Madison, Wiscon. These commercially available vectors, e.g., pBR322, pSPORT, pBluescriptIISK, pcDNAI, and pcDNAII all have a multiple cloning site into which the chimeric genes of the present invention can be conveniently inserted using conventional recombinant techniques. The constructed expression vectors can be introduced into host cells by various transformation or transfection techniques generally known in the art.

In another embodiment, mammalian cells are used as host cells for the expression of the fusion proteins and detection of protein-protein interactions. For this purpose, virtually any mammalian cells can be used including normal tissue cells, stable cell lines, and transformed tumor cells. Conveniently, mammalian cell lines such as CHO cells, Jurkat T cells, NIH 3T3 cells, HEK-293 cells, CV-1 cells, COS-1 cells, HeLa cells, VERO cells, MDCK cells, WI38 cells, and the like are used. Mammalian expression vectors are well known in the art and many are commercially available. Examples of suitable promoters for the transcription of the chimeric genes in mammalian cells include viral transcription promoters derived from adenovirus, simian virus 40 (SV40) (e.g., the early and late promoters of SV40), Rous Sarcoma virus (RSV), and cytomegalovirus (CMV) (e.g., CMV immediate-early promoter), human immunodeficiency virus (HIV) (e.g., long terminal repeat (LTR)), vaccinia virus promoter (e.g., 7.5K promoter), and herpes simplex virus (HSV) (e.g., thymidine kinase promoter). Inducible promoters can

also be used. Suitable inducible promoters include, for example, the tetracycline responsive element (TRE) (*See* Gossen *et al., Proc. Natl. Acad. Sci. USA,* 89:5547-5551 (1992)), metallothionein IIA promoter, ecdysone-responsive promoter, and heat shock promoters. Suitable origin of replication for the replication and maintanence of the expression vectors in mammalian cells include, e.g., the Epstein Barr origin of replication in the presence of the Epstein Barr nuclear antigen (*see* Sugden *et al., Mole. Cell. Biol.,* 5:410-413 (1985)), and the SV40 origin of replication in the presence of the SV40 T antigen (which is present in COS-1 and COS-7 cells) (*see* Margolskee *et al., Mole. Cell. Biol.*, 8:2837 (1988)). Suitable selection markers include, but are not limited to, genes conferring resistance to neomycin, hygromycin, zeocin, and the like. Many commercially available mammalian expression vectors may be useful for the present invention, including, e.g., pCEP4, pcDNAI, pIND, pSecTag2, pVAX1, pcDNA3.1, and pBI-EGFP, and pDisplay. The vectors can be introduced into mammalian cells using any known techniques such as calcium phosphate precipitation, lipofection, electroporation, and the like. The bait vector and prey vector can be co-transformed into the same cell, or alternatively, introduced into two different cells which are subsequently fused together by cell fusion or other suitable techniques.

Viral expression vectors can also be used for the expression of the fusion proteins. Typically, viral vectors having the chimeric genes incorporated therein are viable and can be easily introduced into host cells by viral infection. Viral expression vectors generally known in the art include viral vectors based on adenovirus, bovine papilloma virus, murine stem cell virus (MSCV), MFG virus, and retrovirus. *See* Sarver, *et al., Mol. Cell. Biol.*, 1: 486 (1981); Logan & Shenk, *Proc. Natl. Acad. Sci. USA*, 81:3655-3659 (1984); Mackett, *et al., Proc. Natl. Acad. Sci. USA*, 79:7415-7419 (1982); Mackett, *et al., J. Virol.,* 49:857-864 (1984); Panicali, *et al., Proc. Natl. Acad. Sci. USA,* 79:4927-4931 (1982); Cone & Mulligan, *Proc. Natl. Acad. Sci. USA*, 81:6349-6353 (1984); Mann *et al., Cell*, 33:153-159 (1993); Pear *et al., Proc. Natl. Acad. Sci. USA*, 90:8392-8396 (1993); Kitamura *et al., Proc. Natl. Acad. Sci. USA*, 92:9146-9150 (1995); Kinsella *et al., Human Gene Therapy,* 7:1405-1413 (1996); Hofmann *et al., Proc. Natl. Acad. Sci. USA*, 93:5185-5190 (1996); Choate *et al., Human Gene Therapy*, 7:2247 (1996); WO

94/19478; Hawley *et al., Gene Therapy*, 1:136 (1994) and Rivere *et al., Genetics*, 92:6733 (1995), all of which are incorporated by reference.

Generally, to construct a viral vector, a chimeric gene according to the present invention can be operably linked to a suitable promoter. The promoter-chimeric gene construct is then inserted into a non-essential region of the viral vector, typically a modified viral genome. This results in a viable recombinant virus capable of expressing the fusion protein encoded by the chimeric gene in infected host cells. Once in the host cell, the recombinant virus typically is integrated into the genome of the host cell. However, recombinant bovine papilloma viruses typically replicate and remain as extrachromosomal elements.

In another embodiment, the detection assays of the present invention are conducted in plant cell systems. Methods for expressing exogenous proteins in plant cells are well known in the art. *See generally,* Weissbach & Weissbach, *Methods for Plant Molecular Biology*, Academic Press, NY, 1988; Grierson & Corey, *Plant Molecular Biology*, 2d Ed., Blackie, London, 1988. Recombinant virus expression vectors based on, e.g., cauliflower mosaic virus (CaMV) or tobacco mosaic virus (TMV) can all be used. Alternatively, recombinant plasmid expression vectors such as Ti plasmid vectors and Ri plasmid vectors are also useful. The chimeric genes encoding the fusion proteins of the present invention can be conveniently cloned into the expression vectors and placed under control of a viral promoter such as the 35S RNA and 19S RNA promoters of CaMV or the coat protein promoter of TMV, or of a plant promoter, e.g., the promoter of the small subunit of RUBISCO and heat shock promoters (e.g., soybean hsp17.5-E or hsp17.3-B promoters).

In addition, the *in vivo* assay of the present invention can also be conducted in insect cells, e.g., *Spodoptera frugiperda* cells, using a baculovirus expression system. Expression vectors and host cells useful in this system are well known in the art and are generally available from various commercial vendors. For example, the chimeric genes of the present invention can be conveniently cloned into a non-essential region (e.g., the polyhedrin gene) of an *Autographa californica* nuclear polyhedrosis virus (AcNPV) vector and placed under control of an AcNPV promoter (e.g., the polyhedrin promoter). The non-occluded recombinant viruses thus generated can be used to infect host cells

such as *Spodoptera frugiperda* cells in which the chimeric genes are expressed. *See* Smith, U.S. Patent No. 4,215,051.

In a preferred embodiment of the present invention, the fusion proteins are expressed in a yeast expression system using yeasts such as *Saccharomyces cerevisiae, Hansenula polymorpha, Pichia pastoris,* and *Schizosaccharomyces pombe* as host cells. The expression of recombinant proteins in yeasts is a well developed area, and the techniques useful in this respect is disclosed in detail in *The Molecular Biology of the Yeast Saccharomyces*, Eds. Strathern *et al.*, Vols. I and II, Cold Spring Harbor Press, 1982; Ausubel *et al., Current Protocols in Molecular Biology,* New York, Wiley, 1994; and Guthrie and Fink, *Guide to Yeast Genetics and Molecular Biology,* in *Methods in Enzymology*, Vol. 194, 1991, all of which are incorporated herein by reference. Sudbery, *Curr. Opin. Biotech.*, 7:517-524 (1996) reviews the success in the art in expressing recombinant proteins in various yeast species, the entire content and references cited therein are incorporated herein by reference. In addition, Bartel and Fields, eds., *The Yeast Two-Hybrid System*, Oxford University Press, New York, NY, 1997 contains extensive discussions of recombinant expression of fusion proteins in yeasts in connection with various yeast two-hybrid systems, and cites numerous relevant references. These and other methods known in the art can all be used for purposes of the present invention. The application of such methods to the present invention should be apparent to a skilled artisan apprised of the present disclosure.

Generally, each of the two chimeric genes (one having an N-intein coding sequence and the other having a C-intein coding sequence) of the present invention is included into a separate expression vector (bait vector and prey vector). Both vectors can be co-transformed into a single yeast host cell. As will be apparent to a skilled artisan, it is also possible to express both chimeric genes from a single vector. In a preferred embodiment, the bait vector and prey vector are introduced into two haploid yeast cells of opposite mating types, e.g., a-type and α-type, respectively. The two haploid cells can be mated at a desired time to form a diploid cell expressing both chimeric genes.

Generally, the bait and prey vectors for recombinant expression in yeasts include a yeast replication origin such as the 2 μ origin or the *ARS4* sequence for the replication and maintenance of the vectors in yeast cells. Preferably, the vectors also have a bacteria

origin of replication (e.g., colE1) and a bacteria selection marker (e.g., amp^R marker, i.e., *bla* gene). Optionally, the *CEN6* centromeric sequence is included to control the replication of the vectors in yeast cells. Any constitutive or inducible promoters capable of driving gene transcription in yeast cells may be employed to control the expression of the chimeric genes. Such promoters are operably linked to the chimeric genes. Examples of suitable promoters include but are not limited to, the yeast *ADH1* (alcohol dehydrogenase I) promoter (constitutive), *PGK1* promoter (constitutive), *TEF1* promoter (constitutive), *GPD* promoter (constitutive), *GAL1* promoters (such that the chimeric gene is expressed in the presence of galactose but not glucose), LexA operators (LexA inducible), *HIS3* promoter (constitutive), SPO13 promoter (Gal4p inducible), *CUP1* promoter (Cu^{2+} inducible), *CYC1* promoter, human estrogen receptor gene promoter, AOX/MOX promoter from *H. polymorpha* and *P. Pastoris* (repressed by glucose or ethanol and induced by methanol), and the like. **[PLEASE VERIFY AND/OR MODIFY.]** Inducible promoters are preferred when the fusion proteins encoded by the chimeric genes or the reporter proteins resulting from protein trans-splicing are toxic to the host cells. If it is desirable, certain transcription repressing sequences such as the upstream repressing sequence (URS) from SPO13 promoter can be operably linked to the promoter sequence, e.g., linked to the 5' end of the promoter region. Such upstream repressing sequences function to fine-tune the expression level of the chimeric genes.

Preferably, a transcriptional termination signal is operably linked to the chimeric genes in the vectors. Generally, transcriptional termination signal sequences derived from, e.g., the *CYC1* and *ADH1* genes can be used.

Additionally, it is preferred that the bait vector and prey vector contain one or more selectable markers for the selection and maintenance of only those yeast cells that harbor the chimeric genes of the present invention. Any selectable markers known in the art can be used for purposes of this invention so long as yeast cells expressing the chimeric gene(s) of the present invention can be positively identified or negatively selected. Examples of markers that can be positively selected based on color assays include the lacZ gene which encodes β-galactosidase, the firefly luciferase gene, secreted alkaline phosphatase, horseradish peroxidase, the blue fluorescent protein (BFP), and the green fluorescent protein (GFP) gene (*see* Cubitt *et al.*, *Trends Biochem. Sci.*, 20:448-455

(1995)). Suitable auxotrophic selection markers include, but are not limited to, *URA3*, *HIS3*, *TRP1*, *LEU2*, *LYS2*, *ADE2*, *GUS*, and the like. **[PLEASE VERIFY AND/OR MODIFY.]** Typically, for purposes of auxotrophic selection, the yeast host cells transformed with bait vector and/or prey vector are cultured in a medium lacking a particular nutrient. Antibiotics resistance or sensitivity markers such as chloramphenicol acetyl transferase (CAT) gene, *CAN1* gene (encoding arginine permease which makes cells sensitive to canavanine) (*see* Sikorski *et al.*, *Meth. Enzymol.*, 194:302-318 (1991)), and *CYH2* gene (which confers sensitivity to cycloheximide) (*see* Sikorski *et al.*, *Meth. Enzymol.*, 194:302-318 (1991)) can also be used. In addition, the commonly used *CUP1* gene (encodes metallothionein which confers resistance to copper) is also a suitable selection marker. Each of the above selection markers may be used alone or in combination. One or more selection markers can be included in a particular bait or prey vector. The bait vector and prey vector may have the same or different selection markers. In addition, the selection pressure can be placed on the transformed host cells either before or after mating the haploid yeast cells.

As will be apparent, the selection markers used should complement the host strains in which the bait and/or prey vectors are expressed. In other words, when a gene is used as a selection marker gene, a yeast strain lacking the selection marker gene (or having mutation in the corresponding gene) should be used as host cells. Numerous yeast strains or derivative strains corresponding to various selection markers are known in the art. Many of them have been developed specifically for certain yeast two-hybrid systems. The application and optional modification of such strains with respect to the present invention should be apparent to a skilled artisan apprised of the present disclosure. Methods for genetically manipulating yeast strains using genetic crossing or recombinant mutagenesis are well known in the art. *See e.g.*, Rothstein, *Meth. Enzymol.*, 101:202-211 (1983). By way of example, the following yeast strains are well known in the art, and can be used in the present invention upon necessary modifications and adjustment:

L40 strain which has a genotype *MATa HIS3Δ200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lacZ GAL4*;

AMR70 strain which has a genotype *MATa HIS3 lys2 trp1 leu2 URA3::(lexAop)8-lacZ GAL4*;

EGY48 strain which has a genotype *MATα trp1 his3 ura3 6ops-LEU2;* and

MaV103 strain which has a genotype *MATα leu2-3,112 trp1-901 his3Δ200 ade2-101 gal4Δ gal80Δ SPAL10::URA3 GAL1::lacZ GAL1::HIS3@LYS2 can1^R cyh2^R* (*see* Vidal *et al., Proc. Natl. Acad. Sci. USA,* 93:10315-10320 (1996)). Such strains are generally available in the research community, and can also be obtained by simple yeast genetic manipulation. *See, e.g., The Yeast Two-Hybrid System*, Bartel and Fields, eds., pages 173-182, Oxford University Press, New York, NY, 1997

In addition, the following yeast strains are commercially available:

Y190 strain which is available from Clontech, Palo Alto, California and has the genotype *MATa gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112 URA3::GAL-lacZ, LYS2::GAL(UAS)-HIS3 cyh.r*; and

YRG-2 Strain which is available from Stratagene, La Jolla, California and has the genotype *MATα ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 gal4-542 gal80-538 LYS2::UAS$_{GAL1}$-TATA $_{GAL1}$-HIS3 URA3::UAS$_{GAL4\ 17mers(x3)}$-TATA$_{CYC1}$-lacZ.*

In fact, different versions of vectors and host strains specially designed for yeast two-hybrid system analysis are available in kits from commercial vendors such as Clontech, Palo Alto, California and Stratagene, La Jolla, California, all of which can be modified for use in the present invention.

As described above, each of the two fusion constructs should be designed such that the interaction between the first and second test agents is determinable by detecting or measuring changes in the inactive reporter in the assay system. It will be apparent from the above discussion, the inactive reporter can be any molecules or moieties so long as changes in the reporter that are specifically associated with intein-mediated trans-splicing are detectable. It will be recognized that although the reporters and selection markers can be of similar types and used in a similar manner in the present invention, the reporters and selection markers should be carefully selected in a particular detection assay such that they are distinguishable from each other and do not interfere with each other's function.

Conveniently, the occurrence of trans-splicing can be detected by detecting changes in the size of the reporter. For example, the sizes of the various components of the fusion constructs can be designed such that the "active reporter" which is generated when the inactive reporter is simply cleaved off from one of the fusion constructs or recombined with one or more other components of the fusion constructs, is distinguishable from the "inactive reporter" and other trans-splicing products based on size, i.e., molecular weight. In both *in vitro* and *in vivo* assays, the inactive reporter can be pre-labeled with, e.g., radioactive isotope or fluorescence or other detectable markers, and the active reporter can be detected in, e.g., gel electrophoresis either before or after purification. Purification can be based on specific affinity columns using an antigen-specific protein, e.g., light-chain immunoglobulin, heavy-chain immunoglobulin, avidin, streptavidin, protein A, and antigenic peptides. Conveniently, the commonly used and commercially available epitope tags may be used as size-based reporters. Such epitope tags include sequences derived from, e.g., influenza virus hemagglutinin (HA), Simian Virus 5 (V5), polyhistidine (6xHis), *c-myc*, lacZ, GST, and the like. For example, proteins with polyhistidine tags can be easily detected and/or purified with Ni affinity columns. One advantage for using such epitope tags is that specific antibodies to many of these epitope tags are generally commercially available. Alternatively, an epitope-specific antibody specifically to the "active reporter" can be used to detect the level of the active reporter generated in the assay without purification.

In another embodiment, the fusion constructs are designed such that the active reporter produced during intein-mediated trans-splicing can be detected by a color-based assay. For example, when an N-terminal portion of the lacZ protein (β-galactosidase) is fused to the N-terminus of an N-intein in a fusion construct and a C-terminal portion of the lacZ protein is fused to the C-terminus of a C-intein in another fusion construct, protein trans-splicing will religate the N- and C-terminal portions of the lacZ protein to form a full-length complete and active lacZ protein. Thus, in the presence of a substrate for β-galactosidase (e.g., X-Gal, i.e., 5-bromo-4-chloro-3-indolyl-β-D-galactoside), the trans-splicing can be detected based on appearance of a blue color or by quantitative colorimetric assay. To produce the chimeric genes in this embodiment of the invention, the lacZ gene encoding β-galactosidase can be divided into a 5' portion and a 3' portion

in any manner to encode an N-terminal portion and a C-terminal portion of the β-galactosidase. As discussed above, it may be advantageous to facilitate protein splicing if the first amino acid immediately following C-intein is cysteine, serine, or threonine. Thus, if at all possible, the division of the lacZ gene is made immediately before a genetic codon for cysteine, serine, or threonine such that the first amino acid in the C-terminal portion of β-galactosidase immediately following a C-intein in a fusion construct is one of the three preferred amino acids. Certain mutations may also be introduced into the lacZ gene to substitute a cysteine, serine or threonine for another amino acid, or for any other purposes, so long as the mutation does not adversely interfere with protein trans-splicing or the detection of the active reporter protein, i.e., β-galactosidase. **[PLEASE VERIFY.]**

As will be apparent, many other reporters can be used in a similar manner in the present invention. Such other reporters include, for example, the green fluorescent protein (GFP), which can be detected by fluorescence assay and sorted by flow-activated cell sorting (FACS) (*See* Cubitt *et al., Trends Biochem. Sci.*, 20:448-455 (1995)), secreted alkaline phosphatase, horseradish peroxidase, the blue fluorescent protein (BFP), and luciferase photoproteins such as aequorin, obelin, mnemiopsin, and berovin (*See* U.S. Patent No. 6,087,476, which is incorporated herein by reference).

In another embodiment, an auxotrophic factor is used as a reporter in an *in vivo* assay in a host strain deficient in the auxotriphic factor. Thus, suitable auxotrophic reporter genes include, but not are limited to, *URA3* gene (encoding orotidine-5'-phosphate decarboxylase which is required for uracil synthesis), *HIS3*gene (encoding imidazole glycerol phosphate decaroxylase which is required for histidine synthesis), *TRP1, LEU2, LYS2, ADE2, GUS,* and the like. For example, yeast cells containing a mutant *URA3* gene can be used as host cells (Ura^- phenotype) for the *in vivo* assay as illustrated in Figure 4. Such cells lack *URA3*-encoded functional orotidine-5'-phsphate decarboxylase, an enzyme required by yeast cells for the biosynthesis of uracil. As a result, the cells are unable to grow on a medium lacking uracil. However, wild-type orotidine-5'-phsphate decarboxylase catalyzes the conversion of a non-toxic compound 5-fluoroorotic acid (5-FOA) to a toxic product, 5-fluorouracil. Thus, yeast cells containing a wild-type *URA3* gene are sensitive to 5-FOA and cannot grow on a medium

containing 5-FOA. Therefore, when an N-terminal portion of the *URA3*-encoded protein (orotidine-5'-phosphate decarboxylase) is fused to the N-terminus of an N-intein in a fusion construct and a C-terminal portion of the *URA3*-encoded protein is fused to the C-terminus of a C-intein in another fusion construct, protein trans-splicing initiated by interaction between the test proteins in the fusion constructs will result in religation of the N- and C-terminal portions of the *URA3*-encoded protein forming a full-length complete and active orotidine-5'-phosphate decarboxylase. This enables the Ura^- Foa^R yeast cells to grow on a uracil deficient medium (Sc-ura plates). However, such cells will not survive on a medium containing 5-FOA. Therefore, protein trans-splicing events and interactions between test proteins can be detected based on cell growth.

Additionally, antibiotic resistance reporters can also be employed in a similar manner. In this respect, host cells sensitive to a particular antibiotics is used. Antibiotics resistance reporters include, for example, chloramphenicol acetyl transferase (CAT) gene, *CAN1* gene (which confers resistance to canavanine), and *CYH2* gene (which confers resistance to cycloheximide). In addition, the commonly used *CUP1* gene (encoding metallothionein which confers resistance to copper) is also a suitable reporter.

In yet another embodiment of the present invention, the fusion constructs are designed such that intein-mediated trans-splicing produces an active reporter that is a transcriptional activator or repressor capable of activating or repressing the expression of a detectable gene. Thus, the trans-splicing event will be detected based on the expression or suppression of the detectable gene. In this embodiment, a "reporting vector" containing the detectable gene operably linked to a transcriptional regulatory sequence is also introduced into the host cells. The above-described selection markers and reporter genes can all be used as detectable gene for this purpose so long as the activation or suppression of the expression of the detectable gene is readily detectable. For example, as illustrated in Figure 5, the *URA3* gene can be used as a detectable gene in connection with either a transcriptional activator or suppressor (An activator is shown in Figure 5). The *URA3* gene is operably linked to a transcriptional regulatory sequence responsive to the transcriptional activator or suppressor. When the active reporter generated in trans-splicing is an activator, the yeast host cells (Ura^-) grow on a uracil deficient (Sc-Ura) medium and the interaction between the test proteins is detected based on yeast colony

formation on the medium. Alternatively, when the active reporter generated in trans-splicing is a suppressor, the yeast host cells (Ura^-) grow on a medium containing 5-fluoroorotic acid (5-FOA). In the absence of an interaction between the test proteins, the *URA3* gene is expressed, and the 5-FOA is converted by the *URA3* gene product into a toxic substance, which inhibits the growth of the host cells. In the presence of an interaction between the test proteins, a suppressor is generated and the *URA3* gene expression is shut off. As a result, yeast colonies can be formed on a medium containing 5-FOA. The transcriptional regulatory sequence is designed such that the detectable gene is specifically responsive to the active reporter. Alternatively, a suitable detectable gene integrated in a chromosome of a host cell can also be used.

Suitable transcription activators include, but are not limited to, GAL4, GCN4, ARD1, the human estrogen receptor, *E. coli* LexA protein, herpes simplex virus VP16 (Triezenberg *et al.*, *Genes Dev.* 2:718-729 (1988)), the *E. coli* B42 protein (acid blob, *see* Gyuris *et al.*, *Cell,* 75:791-803 (1993)), NF-kB p65, and the like. In addition, hybrid transcriptional activators composed of a DNA binding domain from one transcriptional activator and an activation domain from another transcriptional activator are also useful. Examples of transcription suppressors include the Kruppel protein, the engrailed protein, the knirps protein, the paired protein and the even-skipped protein all from Drosophila, the yeast TUP1 protein, the Egr-1 protein, the WT1 protein, the RARa protein, and the KRAB protein, verbA protein, YY1 protein, ADE1B protein, E4B4 protein, SCIP protein, kid-1 protein, Znf2 protein, kox-1 protein, and the like. **[PLEASE VERIFY AND/OR MODIFY.]** The corresponding transcriptional elements specifically interacting with the transcriptional activators or repressors are well known in the art. *See. e.g.,* Hanna-Rose and Hansen, *Trends. Genet.*, 12:229-234 (1996).

Thus, a transcriptional activator or repressor protein can be divided into an N-terminal portion and a C-terminal portion which are fused to the N-terminus of N-intein and C-terminus of C-intein, respectively. Upon protein trans-splicing, a full-length protein emerges as a functional transcriptional activator or repressor which subsequently activates or represses the expression of the detectable gene in the reporting vector. *See* Figure 5. It is recognized that the interaction between the test proteins may bring the two portions of the transcriptional activator or suppressor together which may be sufficient to

initiate or suppress the transcription of the detectable gene. In this respect, this specific embodiment of the present invention may be similar to the classic yeast two-hybrid system. However, unlike the classic transcription-based yeast two-hybrid system, it is possible in the present invention to produce a complete and free active transcriptional activator or suppressor. Thus, the fusion proteins need not be transported into cell nucleus, since the transcriptional activator or suppressor, once formed during protein trans-splicing, is free to translocate into any compartments of the host cell including cell nucleus. Apparently, the test proteins can even be membrane bound proteins. **[PLEASE VERIFY AND/OR MODIFY.]**

The method of the present invention for detecting protein-protein interactions can also be used to screen an expression library or applied in the so-called "interaction mating." Methods for constructing activation domain or DNA binding domain fusion libraries and the use thereof in yeast two-hybrid system are well known in the art and are disclosed in e.g., Vojtek *et al.*, in *The Yeast Two-Hybrid System*, Bartel and Fields, eds., pages 29-42, Oxford University Press, New York, NY, 1997; Zhu *et al.*, in *The Yeast Two-Hybrid System*, Bartel and Fields, eds., pages 73-96, Oxford University Press, New York, NY, 1997. Interaction mating is disclosed in U.S. Patent Nos. 6,057,101 and 6,083,693; and Finley and Brent, in *The Yeast Two-Hybrid System*, Bartel and Fields, eds., pages 197-214, Oxford University Press, New York, NY, 1997. The methods described in the above references can all be applied to the present invention upon appropriate modifications. By way of example, N-intein fusion libraries can be prepared using an expression vector containing a 5' portion of a reporter gene operably linked to the 5' end of N-intein coding sequence. Operably linked to the 3' end of the N-intein coding sequence is a multiple cloning site into which various random or predetermined (e.g., cDNAs) DNA sequences can be inserted in frame. The DNA library thus prepared can be transformed into appropriate yeast cells. In this yeast library, an array of fusion proteins can be expressed each fusion protein containing an N-terminal portion of the reporter protein fused to the N-terminus of the N-intein and a random or predetermined polypeptide fused to the C-terminus of the N-intein. Appropriate yeast cells expressing a fusion protein including a bait protein fused to the N-terminus of a C-intein and the C-terminal portion of the reporter protein fused to the C-terminus of the C-intein can be

used to screen the yeast N-intein fusion library to identify prey proteins capable of interacting with the bait protein.

C-intein fusion libraries can also be established and used in "interaction mating" with the N-intein fusion libraries. In this way, interacting protein pairs can be identified and genes encoding such proteins are isolated.

In yet another embodiment of the detection method of the present invention, the detection assay is used to detect interactions between three or more agents in a trimeric or higher order complex. *See* U.S. Patent No. 5,695,941; Chang *et al., Cell*, 79:131-141 (1994); Tirode *et al., J. Biol. Chem.*, 272:22995-22999 (1997); Van Criekinge *et al., Anal. Biochem.*, 263:62-66 (1998); and Pause *et al., Porc. Natl. Acad. Sci. USA*, 96:9533-9538 (1999), all of which are incorporated herein by reference. Essentially, the above-described detection assay of this invention involving two fusion constructs is conducted in the presence of one or more other test agents. In this manner, interactions between the two test agents in the fusion constructs that require the participation of the other test agents can be detected.

The other test agents can be small molecule ligands that interact with the test agents in the fusion constructs. Many protein-protein interactions require the presence of a small molecule ligand, which becomes an integral part of the assembly formed by the protein interactions. *See* Berlin, in *The Yeast Two-Hybrid System*, Bartel and Fields, eds., pages 259-272, Oxford University Press, New York, NY, 1997. For example, immune suppressants such as cyclosporin A (CsA), FK506, and rapamycin are known to bind with high affinity to immunophilins forming protein-drug complexes which, in turn, bind to specific target proteins to inhibit their activities. Classic yeast two-hybrid system has been employed successfully to isolate proteins interacting with the FKBP12/rapamycin complex. *See, e.g.*, Chiu *et al., Proc. Nat. Acad. Sci. USA*, 91:12574-12578 (1994). A multi-hybrid assay in accordance with the present invention can be conducted both *in vitro* and *in vivo*. In an *in vitro* assay, the small molecule ligands are simply added to the above-described intein-based two-hybrid assay system of the present invention. In an *in vivo* assay, it is necessary that the small molecule ligands are taken-up by the host cells. While many host cells are able to take up various small molecule ligands, certain host cells can also be manipulated to increase the uptake of small molecule ligands. For

example, yeast high uptake mutant such as the *erg6* mutant strains can facilitate the uptake of the test compounds by yeast cells. *See* Gaber *et al., Mol. Cell. Biol.*, 9:3447-3456 (1989).

Many protein interactions require the participation of other proteins. Thus, the other test agents in the multi-hybrid assay of the present invention can also be proteins. Accordingly, genes encoding test proteins other than those in the intein-containing fusion constructs can be co-expressed in host cells with the chimeric genes as described above. Such additional genes may be incorporated into one of the bait or prey vector or the reporting vector. Alternatively, they can be expressed in separate vectors under control of a constitutive or inducible promoter.

In a specific embodiment, the additional test proteins are enzymes capable of post-translationally modifying at least one of the test polypeptides in the intein-containing fusion constructs of the present invention. *See* Figure 6. This is especially useful when one or both of the test proteins in the intein-containing fusion proteins are believed to contain consensus sequences for certain modifying enzymes. A two-hybrid system involving modifying enzymes has been disclosed in, e.g., U.S. Patent No. 5,637,463, which is incorporated herein by reference. This system can be applied to the present invention upon appropriate modifications as will be apparent to a skilled artisan apprised of the present disclosure. Examples of useful modifying enzymes include protein kinases which catalyze protein phosphorylation (e.g., serine/threonine phosphorylation, tyrosine phosphorylation by tyrosine kinase, s*ee* Lioubin *et al., Genes Dev.*, 10:1084-1095 (1996)); Keegan *et al., Oncogene,* 12:1537-1544 (1996), fatty acid acylation, ADP-ribosylation, myristylation, and glycosylation. In an *in vivo* assay, the modifying enzymes can be co-expressed in the host cells with the intein-containing fusion proteins. It is recognized that over-expression of certain modifying enzymes such as tyrosine kinases may be toxic to host cells. This can be avoided by using inducible promoters or weak promoters to drive the expression of the toxic modifying enzymes in host cells.

As discussed above, the detection method of the present invention is especially useful in detecting interactions between extracellular proteins, which has not been achieved by conventional two-hybrid systems known in the art. As shown in Figure 7, to

apply the present invention to detecting extracellular protein interactions, the intein-containing fusion proteins are designed to include a membrane anchoring domain (MAD) and optionally a signal peptide such that the test proteins in the fusion proteins are exposed to extracellular environment but anchored on cell membrane by the membrane anchoring domains. At the same time, the N-intein and C-intein are retained within the host cell and intein-mediated protein trans-splicing can occur within the host cell generating an active reporter protein.

Many protein domains functioning to anchor proteins to cell membrane are known in the art and can all be used for purposes of the present invention. For example, the membrane anchoring domain can be a transmembrane domain derived from a known protein or an artificial sequence of hydrophobic amino acid residues sufficient to effect transmembrane spanning. Alternatively, an amino acid sequence containing a consensus sequence for post-translational modification, e.g., the covalent attachment of lipid molecules, can also be used. In addition, the membrane anchoring domain can also be a polypeptide that exhibits sufficient affinity to a cell surface protein or cell membrane component to effect binding of the molecule to the surface of the cell membrane.

Alternatively, fusion constructs are designed to have a signal peptide or a secretion signal for protein translocation but lack a membrane anchoring domain such that the fusion proteins are secreted into the extracellular environment to allow the detection assay to be conducted *in vitro* without having to purify the fusion proteins.

In accordance with another aspect of the present invention, a method is also provided for identifying a compound capable of modulating an interaction between interacting test agents including proteins. By "modulating" or "modulation" it is intended to mean that the compound interferes with, weakens, dissociates or disrupt particular protein-protein interactions, or alternatively, initiates, facilitates or stabilizes particular protein-protein interactions.

As discussed above, most proteins exercise their cellular functions through their interactions with other proteins. Protein-protein interactions form the basis of almost all biological processes. Each biological process or cell machine is composed of a network of interacting proteins. For example, many enzymatic reactions are associated with large protein complexes formed by interactions among enzymes, protein substrates and protein

modulators. In addition, protein-protein interactions are also part of the mechanism for signal transduction and other basic cellular functions such as cell cycle regulation, gene transcription, and translation. Undoubtedly, protein-protein interactions are involved in various disease pathways. Thus, compounds that modulate particular protein-protein interactions in disease pathways are potential therapeutic agents useful in treating or preventing diseases. In this respect, both compounds capable of interfering with undesirable protein-protein interactions and compounds that trigger or stabilize desirable protein-protein interactions can be useful.

The intein-based system of the present invention is especially suited for screening such compounds. The screen assay in accordance with the present invention can be conducted either *in vitro* or *in vivo* using bacterial cells, yeasts, insect cells or animal cells as host cells. As will be apparent, the screen assay can be based on any of the above-described embodiments of the intein-based method for detecting protein-protein interaction. Thus, two proteins whose interaction needs to be modulated are used as test proteins in the intein-containing fusion constructs of the present invention. The two fusion constructs containing N-intein and C-intein respectively are allowed to interact with each other in the presence of a test compound, and the ability of the test compound to modulate the interaction between the two known proteins is determined by detecting the presence or absence of an active reporter or measuring the relative level of the active reporter.

The screen assay of the present invention can be used to identify compounds capable of triggering or stabilizing particular protein-protein interactions. As is known in the art, many protein-protein interactions require the presence of small molecule ligands or other proteins. For example, immune suppressants such as cyclosporin A (CsA), FK506, and rapamycin are known to exert their therapeutic effect by mediating the binding of immunophilins to specific target proteins. Thus, two proteins whose interaction needs be initiated or strengthened by a therapeutic compound are used as test proteins in the intein-based two-hybrid system of the present invention. The fusion proteins are expressed and allowed to interact with each other in the presence of one or more test compounds. In an *in vivo* assay, e.g., in a yeast system, a positively selected marker is preferably used as a reporter. In this manner, a detectable signal (e.g.,

appearance of color or fluorescence, or cell survival) is present only if the test compound is able to mediate the interaction between the two test proteins.

The screen assay of the present invention is also useful in identifying compounds capable of interfering with or disrupting particular protein-protein interactions. For example, inhibitors of interactions between pathogen coat proteins and their corresponding receptors on human cell surface may be selected by the screen assay. Such inhibitors are potential preventive or therapeutic agents against the pathogen. In another example, compounds capable of dissociating interactions between oncogene products and their cellular targets are potential anti-cancer agents. Again, two proteins of interest whose interaction needs be disrupted by a therapeutic compound are used as test proteins in the intein-based two-hybrid system of the present invention. The fusion proteins are expressed and allowed to interact with each other in the presence of one or more test compounds.

In a preferred embodiment, a counterselectable marker is used as a reporter such that a detectable signal (e.g., appearance of color or fluorescence, or cell survival) is present only when the test compound is capable of interfering with the interaction between the two test proteins. In this respect, the reporters used in various "reverse two-hybrid systems" known in the art can be selected. Reverse two-hybrid systems are disclosed in, e.g., U.S. Patent Nos. 5,525,490; 5,733,726; 5,885,779; Vidal *et al., Proc. Natl. Acad. Sci. USA,* 93:10315-10320 (1996); and Vidal *et al., Proc. Natl. Acad. Sci. USA,* 93:10321-10326 (1996), all of which are incorporated herein by reference.

Examples of suitable counterselectable reporters useful in a yeast system include the *URA3* gene (encoding orotidine-5'-decarboxylase which converts 5-fluroorotic acid (5-FOA) to 5-fluorouracil which is toxic to yeast cells), the *CAN1* gene (encoding arginine permease which transports toxic arginine analog canavanine into yeast cells inhibiting cell growth), the *GAL1* gene (encoding galactokinase which catalyzes the conversion of 2-deoxygalactose to toxic 2-deoxygalactose-1-phosphate which inhibits yeast cell growth), the *LYS2* gene (encoding α-aminoadipate reductase which causes yeast cells unable to grow on a medium containing α-aminoadipate as the sole nitrogen source), the *MET15* gene (encoding O-acetylhomoserine sulfhydrylase which confers on yeast cells sensitivity to methyl mercury), and the *CYH2* gene (encoding L29 ribosomal

protein which confers sensitivity to cycloheximide). In addition, any known cytotoxic agents including cytotoxic proteins such as the diphtheria toxin (DTA) catalytic domain can also be used as counterselectable reporters. *See* U.S. Patent No. 5,733,726. DTA causes the ADP-ribosylation of elongation factor-2, and thus inhibits protein synthesis and causes cell death. Other examples of cytotoxic agents include recin, Shiga toxin, and exotoxin A of *Pseudomonas aeruginosa.*

For example, when the *URA3* gene is used as a counterselectable reporter gene, yeast cells containing a mutant *URA3* gene can be used as host cells (Ura^- Foa^R phenotype) for the *in vivo* assay. Such cells lack *URA3*-encoded functional orotidine-5'-phsphate decarboxylase, an enzyme required for the biosynthesis of uracil. As a result, the cells are unable to grow on media lacking uracil. However, because of the absence of a wild-type orotidine-5'-phsphate decarboxylase, the yeast cells cannot convert non-toxic 5-fluoroorotic acid (5-FOA) to a toxic product, 5-fluorouracil. Thus, such yeast cells are resistant to 5-FOA and can grow on a medium containing 5-FOA. Therefore, to screen for a compound capable of disrupting interaction between protein X and protein Y, the above-described intein-based system is utilized in which one fusion protein has an N-terminal portion of the *URA3*-encoded protein (orotidine-5'-phosphate decarboxylase) fused to the N-terminus of an N-intein and protein X fused to the C-terminus of the N-intein. Another fusion protein contains a C-terminal portion of the *URA3*-encoded protein fused to the C-terminus of a C-intein and protein Y fused to the N-terminus of the C-intein. After the fusion proteins are expressed in the Ura^- Foa^R yeast cells, an *in vivo* screen assay can be conducted in the presence of a test compound with the yeast cells being cultured on a medium containing uracil and 5-FOA. If the test compound does not interrupt the interaction between protein X and protein Y, intein-mediated trans-splicing produces an active *URA3* gene product, i.e., orotidine-5'-decarboxylase, which converts 5-FOA to toxic 5-fluorouracil. As a result, the yeast cells cannot grow. On the other hand, when the test compound interrupts the interaction between protein X and protein Y, intein-mediated trans-splicing does not occur and no active orotidine-5'-decarboxylase is produced in the host yeast cells. Consequently, the yeast cells will survive and grow on the 5-FOA-containing medium. Therefore, compounds capable of interfering with or

dissociating particular protein-protein interactions can thus be identified based on colony formation.

As will be apparent, the screen assay of the present invention can be applied in a large-scale screening. For example, combinatorial technologies can be employed to construct combinatorial libraries of small organic molecules or small peptides. *See generally, e.g.*, Kenan *et al., Trends Biochem. Sc.*, 19:57-64 (1994); Gallop *et al., J. Med. Chem.*, 37:1233-1251 (1994); Gordon *et al., J. Med. Chem.*, 37:1385-1401 (1994); Ecker *et al., Biotechnology,* 13:351-360 (1995). Such combinatorial libraries of compounds can be applied to the screen assay of the present invention to isolate specific modulators of particular protein-protein interactions. In the case of random peptide libraries, the random peptides can be co-expressed with the fusion proteins of the present invention in host cells and assayed *in vivo*. *See e.g.*, Yang *et al., Nucl. Acids Res.*, 23:1152-1156 (1995). Alternatively they can be added to the host cells for uptake. Since peptides are generally not easy for cells to absorb, *in vitro* screen assays may be preferably. Similarly, phage display libraries can also be constructed and screened in an *in vitro* assay in accordance with the present invention.

Conveniently, yeast host cells are used in an *in vivo* screen assay. For example, haploid cells of MATa mating type expressing an N-intein-containing fusion protein as described above is mated with haploid cells of MATα mating type expressing the other fusion protein containing a C-intein. Upon mating, the diploid cells are spread on a suitable medium to form a lawn. Drops of test compounds can be deposited onto different areas of the lawn. After culturing the lawn for an appropriate period of time, drops containing a compound capable of modulating the interaction between the particular test proteins in the fusion proteins can be identified based on a ring of growth formed around the drops.

The screen assays of the present invention for identifying compounds capable of modulating protein-protein interactions can also be fine-tuned by various techniques to adjust the thresholds or sensitivity of the positive and negative selections. For example, mutations can be introduced into the reporter proteins to adjust their activities. The uptake of test compounds by the host cells can also be adjusted. For example, yeast high uptake mutants such as the *erg6* mutant strains can facilitate yeast uptake of the test

compounds. *See* Gaber *et al., Mol. Cell. Biol.*, 9:3447-3456 (1989). Likewise, the uptake of the selection compounds such as 5-FOA, 2-deoxygalactose, cycloheximide, α-aminoadipate, and the like can also be fine-tuned.

Once an effective compound is identified, structural analogs or mimetics thereof can be produced based on rational drug design with the aim of improving drug efficacy and stability, and reducing side effects. Methods known in the art for rational drug design can be used in the present invention. *See, e.g.,* Hodgson *et al., Bio/Technology,* 9:19-21 (1991); U.S. Patent Nos. 5,800,998 and 5,891,628, all of which are incorporated herein by reference. An example of rational drug design is the development of HIV protease inhibitors. *See* Erickson *et al., Science,* 249:527-533 (1990).

Preferably, structural information on the protein-protein interaction to be modulated is obtained. For example, each of the interacting pair can be expressed and purified. The purified interacting protein pairs are then allowed to interact with each other *in vitro* under appropriate conditions. Optionally, the interacting protein complex can be stabilized by crosslinking or other techniques. The interacting complex can be studied using various biophysics techniques including, e.g., X-ray crystallography, NMR, computer modeling, mass spectrometry, and the like. Likewise, structural information can also be obtained from protein complexes formed by interacting proteins and a compound that initiates or stabilizes the interaction of the proteins.

In addition, understandings of the interaction between the proteins of interest in the presence or absence of a modulating compound can also be derived from mutagenesis analysis using the above-described detection method of the present invention. Indeed, the detection method of this invention is particularly useful in analyzing and characterizing protein-protein interactions. In this respect, various mutations can be introduced into the interacting proteins and the effect of the mutations on protein-protein interaction is examined by the above-discussed detection method.

Various mutations including amino acid substitutions, deletions and insertions can be introduced into a protein sequence using conventional recombinant DNA technologies. Generally, it is particularly desirable to decipher the protein binding sites. Thus, it is important that the mutations introduced only affect protein-protein interaction and cause minimal structural disturbances. Mutations are preferably designed based on

any known information on the three-dimensional structure of the interacting proteins. Preferably, mutations are introduced to alter charged amino acids or hydrophobic amino acids exposed on the surface of the proteins, since ionic interactions and hydrophobic interactions are often involved in protein-protein interactions. Alternatively, the "alanine scanning mutagenesis" technique is used. *See* Wells, *et al., Methods Enzymol.*, 202:301-306 (1991); Bass *et al., Proc. Natl. Acad. Sci. USA*, 88:4498-4502 (1991); Bennet *et al., J. Biol. Chem.*, 266:5191-5201 (1991); Diamond *et al., J. Virol.*, 68:863-876 (1994). Using this technique, charged or hydrophobic amino acid residues of the interacting proteins are replaced by alanine, and the effect on the interaction between the proteins is analyzed using the above-described detection method. For example, the entire protein sequence can be scanned in a window of five amino acids. When two or more charged or hydrophobic amino acids appear in a window, the charged or hydrophobic amino acids are changed to alanine using standard recombinant DNA techniques. The thus mutated proteins are used as "test proteins" in the above-described detection method to examine the effect of the mutations on protein-protein interaction. Preferably, the mutagenesis analysis is conducted both in the presence and in the absence of an identified modulating compound. In this manner, the domains or residues of the proteins important to protein-protein interaction and/or the interaction between the modulating compound and the proteins can be identified.

Based on the structural information obtained, structural relationships between the interacting proteins as well as between the identified compound and the interacting proteins are elucidated. The moieties and the three-dimensional structure of the identified compound, i.e., lead compound, critical to its modulating effect on the interaction of the known proteins of interest are revealed. Medicinal chemists can then design analog compounds having similar moieties and structures.

In addition, an identified peptide compound capable of modulating particular protein-protein interactions can also be analyzed by the alanine scanning technique to determine the domains or residues of the peptide important to its modulating effect on particular protein-protein interactions. The peptide compound can be used as a lead molecule for rational design of small organic molecules. *See* Huber *et al., Curr. Med. Chem.*, 1:13-34 (1994).

The residues or domains critical to the modulating effect of the identified compound constitute the active region of the compound known as its "pharmacophore." Once the pharmacophore has been elucidated, a structural model can be established by a modeling process which may include analyzing the physical properties of the pharmacophore such as stereochemistry, hydrophobicity, charge, bonding, and size using data from a range of sources, e.g., NMR analysis, X-ray diffraction data, alanine scanning, and spectroscopic techniques and the like. Various techniques including computational analysis, similarity mapping and the like can all be used in this modeling process. *See e.g.,* Perry *et al., in OSAR: Quantitative Structure-Activity Relationships in Drug Design*, pp.189-193, Alan R. Liss, Inc., 1989; Rotivinen *et al., Acta Pharmaceutical Fennica,* 97:159-166 (1988); Lewis *et al., Proc. R. Soc. Lond.*, 236:125-140 (1989); McKinaly *et al., Annu. Rev. Pharmacol. Toxiciol.*, 29:111-122 (1989). Commercial molecular modeling systems available from Polygen Corporation, Waltham, MA, include the CHARMm program, which performs the energy minimization and molecular dynamics functions, and QUANTA program which performs the construction, graphic modeling and analysis of molecular structure. Such programs allow interactive construction, visualization and modification of molecules. Other computer modeling programs are also available from BioDesign, Inc. (Pasadena, CA.), Hypercube, Inc. (Cambridge, Ontario), and Allelix, Inc. (Mississauga, Ontario, Canada).

A template can be formed based on the established model. Various compounds can then be designed by linking various chemical groups or moieties to the template. Various moieties of the template can also be replaced. In addition, in the case of a peptide lead compound, the peptide or mimetics thereof can be cyclized, e.g., by linking the N-terminus and C-terminus together, to increase its stability. These rationally designed compounds are further tested. In this manner, pharmacologically acceptable and stable compounds with improved efficacy and reduced side effect can be developed. The compounds identified in accordance with the present invention can be incorporated into a pharmaceutical formulation suitable for administration into an individual.

In yet another aspect of the present invention, a kit is provided comprising various vectors and reagents described above. The kit will provide users some convenience in practicing the various embodiments of the present invention. In particular, the kit can be

used in detecting and/or characterizing protein-protein interactions, and in screen assays for identifying specific compounds capable of modulating known protein-protein interactions. Accordingly, components includable in the kit will be apparent to a skilled artisan apprised of the present disclosure. Specifically, any vectors, reagents, and the like described above in connection with various embodiments of the present invention can be included in the kit. Typically, the various components of the kit are placed in a rack, compartmentalized support or enclosed container for purposes of organizing and/or transporting the kit.

In a specific embodiment, the kit includes at least a pair of expression vectors. One expression vector contains a chimeric gene operably linked to a transcription regulatory sequence. The chimeric gene includes a DNA sequence encoding an N-intein and a multiple cloning site (MCS). The multiple cloning site is operably linked to the N-intein coding sequence such that a DNA sequence encoding a test polypeptide of interest can be conveniently inserted in frame into the MCS and a fusion protein can be produced containing the N-intein and the test polypeptide. Likewise, the other expression vector also contains a transcription regulatory sequence operably linked to a chimeric gene which includes a DNA sequence encoding a C-intein and a multiple cloning site (MCS). The multiple cloning site is operably linked to the C-intein coding sequence such that a DNA sequence encoding another test polypeptide of interest can be conveniently inserted in frame into the MCS and a fusion protein can be produced containing the C-intein and the test polypeptide. One or both of the chimeric genes further contain an operably linked DNA sequence encoding an inactive reporter protein capable of being converted to an active reporter protein upon trans-splicing mediated by the N-intein and the C-intein. Various arrangements of the chimeric genes can be used, as will apparent from the discussions above in connection with the method for detecting protein-protein interactions of the present invention. In a preferred embodiment, specially selected and/or modified coding sequences for the N-intein and C-intein are used such that the N-intein and C-intein do not significantly interact with one another.

Optionally, the chimeric genes also have DNA sequences encoding membrane anchoring domains and/or signal peptides. Such a kit will be useful in detecting or characterizing interactions between membrane or extracellular proteins, or in screen

assays for identifying compounds modulating interactions between membrane or extracellular proteins.

The expression vectors may also include other components as described above in connection with the bait vectors and prey vectors of the present invention. For example, the expression vectors may contain elements necessary for the replication of the vector in a host cell, the correct transcription and translation of the chimeric gene (e.g., promoters and other transcriptional regulatory elements, transcription termination signal, etc.). The vectors preferably also contain a selection marker gene for selecting and maintaining only those host cells harboring the vectors.

For application in an intein-based multi-hybrid system of the present invention, the kit may further include one or more additional expression vectors each containing a gene encoding a test protein, e.g., a modifying enzyme (e.g., protein kinase, enzymes catalyzing glycosylation, ribosylation, myristalization, etc.). The gene should be placed under control of a constitutive or inducible promoter.

When the reporter protein is a transcription activator or suppressor, the kit may further comprise a reporting vector. As described above, the reporting vector contains a detectable gene under control of a promoter specifically activated or repressed by the activator or suppressor, respectively.

In addition, the kit of the present invention can also comprise one or more types of host cells, for example, yeast host strains for the expression of the chimeric genes and other genes. Preferably, two yeast strains of opposite yeast mating types (MATa and MATα) are provided. The yeast strains should have genotypes suitable for the selection of the various vectors based on the selection marker genes in the vectors, and suitable for the detection of the active reporter generated in the host strains as a result of intein-mediated protein trans-splicing. Optionally, *E. coli* strains for the amplification of the various vectors are also provided in the kit.

Additionally, the kit may also include instructions for using the kit to practice the present invention. The instructions should be in writing in a tangible form or stored in an electronically retrievable medium.

As is apparent from the above description, the present invention provides a powerful versatile intein-based system for detecting and characterizing protein-protein

interactions, and for selecting compounds capable of modulating protein-protein interactions. The system can be used both *in vivo* and *in vitro* with great convenience and can be easily adapted to high-throughput screening procedures. In particular, sensitive genetic selection assays can be conveniently incorporated into the system using host cells such as yeasts, bacteria, and animal cells. Detection of protein-protein interaction is based on intein-mediated protein trans-splicing, which is independent of other cellular factors. As a result, the system is useful in detecting not only interactions between nuclear and cytosolic proteins, but also interactions between membrane and extracellular proteins. In addition, protein trans-splicing typically results in changes in protein structures and functions and formation of free new proteins. As a result, various methods available in the art for detecting changes in protein structures and functions can be incorporated into the system allowing great flexibility in fine tuning and optimizing the system, and adapting the system to various applications.

The present invention will be further described by way of the following examples, which are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

EXAMPLE

A chimeric gene is constructed that encodes a fusion protein consisting of (from the N- to the C-terminus): the N-terminal 154 residues of the 267 amino acid S. cerevisiae Ura3p; 187 N-terminal residues of the VMA1 intein; a flexible linker composed of residues 114 to 163 of IRS1; and residues 31 to 150 of the PI3 kinase catalytic subunit, p110β. A second chimeric gene is constructed that encodes a fusion protein consisting of (from the N- to the C-terminus): residues 427-613 of the PI3 kinase regulatory subunit, p85; the IRS1 linker; 50 C-terminal residues of the VMA1 intein; and the C-terminal 113 residues of Ura3p. Each coding sequence is engineered for expression from the galactose-inducible GAL1 promoter in a yeast expression vector. *Ura3* yeast will be transformed with plasmids that express the two chimeric genes. Transformants are tested for galactose-dependent uracil prototrophy, which indicate association between p110 and p85.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for detecting an interaction between a first test agent and a second test agent, comprising:

providing a first fusion construct and a second fusion construct, said first fusion construct having an N-intein and said first test agent, said second fusion construct having a C-intein and said second test agent, wherein at least one of the two fusion constructs has an inactive reporter capable of being converted to an active reporter upon trans-splicing through said N-intein and said C-intein, and wherein said N-intein and said C-intein do not interact with each other;

allowing said first test agent in said first fusion construct to interact with said second test agent in said second fusion construct; and

detecting said active reporter.

2. The method of Claim 1, wherein said first fusion construct comprises a first said inactive reporter fused to the N-terminus of said N-intein.

3. The method of Claim 2, wherein said first inactive reporter is a non-proteinaceous moiety fused to the N-terminus of said N-intein through an amino acid linker.

4. The method of Claim 2, wherein said first inactive reporter is a polypeptide.

5. The method of Claim 2, wherein the first test agent is fused to the C-terminus of said N-intein.

6. The method of Claim 2, wherein the first test agent is covalently linked to the first inactive reporter.

7. The method of Claim 2, wherein said second fusion construct comprises a second inactive reporter fused to the C-terminus of said C-intein, and wherein an active reporter is formed upon ligation of said first and second inactive reporters.

8. The method of Claim 7, wherein said second inactive reporter is a non-proteinaceous moiety fused to the C-terminus of said C-intein through an amino acid linker selected from the group consisting of cysteine, serine, and threonine.

9. The method of Claim 7, wherein said second inactive reporter is a polypeptide having an N-terminus amino acid selected from the group consisting of cysteine, serine, and threonine.

10. The method of Claim 7, wherein the second test agent is fused to the N-terminus of said C-intein.

11. The method of Claim 7, wherein the second test agent is covalently linked to said second inactive reporter.

12. The method of Claim 1, wherein the first and second fusion constructs are allowed to interact with each other in a substantially cell free environment. (*in vitro*?)

13. The method of Claim 12, wherein said active reporter is detected based on molecular weight.

14. The method of Claim 12, wherein said active reporter is detected by a color assay.

15. The method of Claim 12, wherein said active reporter is detected by an affinity assay.

16. The method of Claim 1, wherein the first and second fusion constructs are allowed to interact with each other in a host cell.

17. The method of Claim 16, wherein said active reporter is an auxotrophic protein and is detectable by cell viability assay.

18. The method of Claim 16, wherein said active reporter is a transcriptional activator or suppressor.

19. The method of Claim 16, wherein said active reporter is detectable based on color.

20. A method for detecting an interaction between a first test agent and a second test agent, comprising:

conducting a detection assay comprising the steps of (a) providing a first fusion construct and a second fusion construct, said first fusion construct having an N-intein and said first test agent, said second fusion construct having a C-intein and said second test agent, wherein at least one of the two fusion constructs has an inactive reporter capable of being converted to an active reporter upon trans-splicing through said N-intein and said C-intein; (b) allowing said first test agent in said first fusion construct to interact with said second test agent in said second fusion construct; and (c) detecting said active reporter;

conducting a control assay in which the interaction between the first and second test agents in said fusion constructs in said detection assay is pre-empted; and

comparing the level of said active reporter in said detection assay and said control assay.

21. The method of Claim 20, wherein said control assay comprises:

allowing said first test agent in said first fusion construct to interact with said second test agent in said second fusion construct in the presence of an inhibitor of said interaction; and

detecting the active reporter.

22. The method of Claim 21, wherein said inhibitor is the first or second test agent.

23. The method of Claim 20, wherein said control assay comprises the steps of:

providing a third fusion construct and a fourth fusion construct, wherein said third fusion construct is same as said first fusion construct except that said third fusion construct has a third test agent but not said first test agent, said fourth fusion construct is same as said second fusion construct except that said fourth fusion construct has a fourth test agent but not said second test agent, and wherein said third and fourth test agents do not interact with each other;

allowing said third fusion construct to interact with said fourth fusion construct; and

detecting said active reporter.

24. The method of Claim 20, wherein said control assay comprises the steps of:

providing a third fusion construct and a fourth fusion construct, wherein said third fusion construct is same as said first fusion construct except that said third fusion construct lacks said first test agent, said fourth fusion construct is same as said second fusion construct except that said fourth fusion construct lacks said second test agent;

allowing said third fusion construct to interact with said fourth fusion construct; and

detecting said active reporter.

25. A method for detecting protein-protein interaction between a first test polypeptide and a second test polypeptide, comprising:

introducing into a host cell a first chimeric gene and a second chimeric gene, said first chimeric gene encoding a first fusion protein having said first test polypeptide, an N-

intein, and a first inactive reporter polypeptide fused to the N-terminus of an N-intein, said second chimeric gene encoding a second fusion protein having said second test polypeptide, a C-intein, and a second inactive reporter polypeptide fused to the C-terminus of said C-intein, wherein ligation between the C-terminus of said first inactive reporter polypeptide and the N-terminus of said second inactive reporter polypeptide forms an active reporter, and wherein said N-intein and said C-intein are incapable of interacting with each other;

expressing said first fusion protein and said second fusion protein in said host cell; and

detecting said active reporter protein.

26. The method of Claim 25, wherein said first inactive reporter polypeptide is an N-terminal fragment of said active reporter protein and said second inactive reporter polypeptide is the remaining C-terminal fragment of said active reporter protein.

27. The method of Claim 25, wherein said host cell is a Saccharomyces cell.

28. The method of Claim 27, wherein said Saccharomyces cell is a diploid cell and said step of introducing into said host cell said first chimeric gene and said second chimeric gene comprises mating a first haploid Saccharomyces cell having said first chimeric gene with a second haploid Saccharomyces cell having said second chimeric gene.

29. The method of Claim 25, wherein said host cell is a mammalian cell.

30. The method of Claim 25, wherein said host cell is a bacteria cell.

31. The method of Claim 25, wherein said first test polypeptide is fused to the C-terminus of said N-intein in said first fusion protein, and said second test polypeptide is fused to the N-terminus of said C-intein in said second fusion protein.

32. The method of Claim 25, wherein said first test polypeptide is fused to the N-terminus of said first inactive reporter polypeptide in said first fusion protein, and said second test polypeptide is fused to the N-terminus of said C-intein in said second fusion protein.

33. The method of Claim 25, wherein said first test polypeptide is fused to the C-terminus of said N-intein in said first fusion protein, and said second test polypeptide is fused to the C-terminus of said second inactive reporter polypeptide in said second fusion protein.

34. The method of Claim 25, wherein said first test polypeptide is fused to the N-terminus of said first inactive reporter polypeptide in said first fusion protein, and said second test polypeptide is fused to the C-terminus of said second inactive reporter polypeptide in said second fusion protein.

35. The method of Claim 25, wherein said active reporter protein is detectable by color assay.

36. The method of Claim 35, wherein said active reporter protein is selected from the group consisting of β-galactosidase, luciferase, green fluorescence protein, blue fluorescence protein, alkaline phosphotase, horseradish peroxidase, and derivatives thereof.

37. The method of Claim 25, wherein said active reporter protein is an auxotrophic protein and is detectable by cell viability assay.

38. The method of Claim 25, wherein the expression of at least one of said fusion proteins in said host cell is inducible and occurs only when said host cell is subject to a predetermined condition.

39. The method of Claim 25, wherein said reporter protein is a transcription activator and said host cell further comprises a detectable gene that is activated only when said transcription activator is present.

40. The method of Claim 25, further comprising expressing a third test polypeptide in said host cell.

41. The method of Claim 40, wherein the interaction between said first and second test polypeptide requires the presence of said third test polypeptide.

42. The method of Claim 41, wherein said third test polypeptide modifies post-translationally at least one of said first and second test polypeptides.

43. The method of Claim 25, further comprising introducing into the host cell a small organic compound to allow said small organic compound to interact with either said first or second test polypeptide or both.

44. The method of Claim 25, wherein each of said first and second fusion proteins has a signal peptide at its N-terminus.

45. The method of Claim 25, wherein each of said first and second fusion proteins further includes a membrane anchoring domain such that said first and second test polypeptides are anchored on cell membrane.

46. The method of Claim 25, wherein each of said first and second fusion proteins further includes a membrane anchoring domain such that said first and second test polypeptides are exposed to an extracellular environment while anchored on cell membrane, and said N-intein, C-intein, and said first and second inactive reporter polypeptides are retained within the host cell.

47. A composition comprising:

a first chimeric gene encoding a first fusion protein comprising an N-intein and a first test polypeptide; and

a second chimeric gene encoding a second fusion protein comprising a C-intein and a second test polypeptide, wherein at least one of the two fusion proteins further include an inactive reporter protein capable of being converted to an active reporter protein upon trans-splicing through said N-intein and said C-intein, and wherein said N-intein and said C-intein are incapable of interacting with each other or with said inactive reporter.

48. The composition of Claim 47, wherein said first fusion protein has an N-terminal fragment of said active reporter protein the C-terminus of which is fused to the N-terminus of said N-intein, wherein said second fusion protein has a C-terminal fragment of the active reporter protein the N-terminus of which is fused to the C-terminus of said C-intein, and wherein, upon trans-splicing, the N-terminal fragment is ligated to the C-terminal fragment thereby forming said active reporter protein or an active derivative thereof.

49. The composition of Claim 47, wherein said first and second chimeric genes are contained in two expression vectors, respectively.

50. The composition of Claim 47, wherein each of said first and second fusion proteins has a signal peptide at its N-terminus and a membrane anchoring domain.

51. A host cell comprising the composition of Claim 47.

52. A host cell comprising the composition of Claim 48.

53. A method for determining whether a compound is capable of modulating an interaction between a first test agent and a second test agent, comprising:

providing a first fusion construct and a second fusion construct, said first fusion construct having an N-intein and said first test agent, said second fusion construct having

a C-intein and said second test agent, wherein at least one of the two fusion constructs has an inactive reporter capable of being converted to an active reporter upon trans-splicing through said N-intein and said C-intein;

allowing said first test agent in said first fusion construct to interact with said second test agent in said second fusion construct in the presence of said compound; and

detecting said active reporter.

54. The method of Claim 53, wherein said N-intein and said C-intein do not interact with each other.

55. The method of Claim 54, wherein said first fusion construct comprises a first said inactive reporter fused to the N-terminus of said N-intein.

56. The method of Claim 55, wherein said first inactive reporter is a non-proteinaceous moiety fused to the N-terminus of said N-intein through an amino acid linker.

57. The method of Claim 55, wherein said first inactive reporter is a polypeptide.

58. The method of Claim 55, wherein the first test agent is fused to the C-terminus of said N-intein.

59. The method of Claim 55, wherein the first test agent is covalently linked to the first inactive reporter.

60. The method of Claim 55, wherein said second fusion construct comprises a second inactive reporter fused to the C-terminus of said C-intein, and wherein an active reporter is formed upon ligation of said first and second inactive reporters.

61. The method of Claim 60, wherein said second inactive reporter is a non-proteinaceous moiety fused to the C-terminus of said C-intein through an amino acid linker selected from the group consisting of cysteine, serine, and threonine.

62. The method of Claim 60, wherein said second inactive reporter is a polypeptide having an N-terminus amino acid selected from the group consisting of cysteine, serine, and threonine.

63. The method of Claim 60, wherein the second test agent is fused to the N-terminus of said C-intein.

64. The method of Claim 60, wherein the second test agent is covalently linked to said second inactive reporter.

65. The method of Claim 53, wherein the first and second fusion constructs are allowed to interact with each other in a substantially cell free environment. (*in vitro*?)

66. The method of Claim 65, wherein said active reporter is detected based on molecular weight.

67. The method of Claim 65, wherein said active reporter is detected by a color assay.

68. The method of Claim 65, wherein said active reporter is detected by an affinity assay.

69. The method of Claim 53, wherein the first and second fusion constructs are allowed to interact with each other in a host cell.

70. The method of Claim 69, wherein said active reporter is an auxotrophic protein and is detectable by cell viability assay.

71. The method of Claim 69, wherein said active reporter is a transcriptional activator or suppressor.

72. The method of Claim 69, wherein said active reporter is detectable based on color.

73. The method of Claim 53, further comprising:

allowing said first test agent in said first fusion construct to interact with said second test agent in said second fusion construct in the absence of said compound;

detecting said active reporter; and

comparing the level of said active reporter determined in the presence and absence of said compound.

74. A method for determining whether a compound is capable of inhibiting an interaction between a first test polypeptide and a second test polypeptide, said first and second test polypeptides are known to interact with each other, said method comprising:

introducing into a host cell a first chimeric gene and a second chimeric gene, said first chimeric gene encoding a first fusion protein having said first test polypeptide, an N-intein, and a first inactive reporter polypeptide fused to the N-terminus of an N-intein, said second chimeric gene encoding a second fusion protein having said second test polypeptide, a C-intein, and a second inactive reporter polypeptide fused to the C-terminus of said C-intein, wherein ligation between the C-terminus of said first inactive reporter polypeptide and the N-terminus of said second inactive reporter polypeptide forms an active reporter;

expressing said first fusion protein and said second fusion protein in said host cell in the presence of said compound; and

detecting said active reporter protein.

75. The method of Claim 74, wherein, said N-intein and said C-intein are incapable of exerting sufficient interaction with each other to mediate protein trans-splicing.

76. The method of Claim 74, wherein said first inactive reporter polypeptide is an N-terminal fragment of said active reporter protein and said second inactive reporter polypeptide is the remaining C-terminal fragment of said active reporter protein.

77. The method of Claim 74, wherein said host cell is a Saccharomyces cell.

78. The method of Claim 77, wherein said Saccharomyces cell is a diploid cell and said step of introducing into said host cell said first chimeric gene and said second chimeric gene comprises mating a first haploid Saccharomyces cell having said first chimeric gene with a second haploid Saccharomyces cell having said second chimeric gene.

79. The method of Claim 74, wherein said host cell is a mammalian cell.

80. The method of Claim 74, wherein said host cell is a bacteria cell.

81. The method of Claim 74, wherein said first test polypeptide is fused to the C-terminus of said N-intein in said first fusion protein, and said second test polypeptide is fused to the N-terminus of said C-intein in said second fusion protein.

82. The method of Claim 74, wherein said first test polypeptide is fused to the N-terminus of said first inactive reporter polypeptide in said first fusion protein, and said second test polypeptide is fused to the N-terminus of said C-intein in said second fusion protein.

83. The method of Claim 74, wherein said first test polypeptide is fused to the C-terminus of said N-intein in said first fusion protein, and said second test polypeptide is

fused to the C-terminus of said second inactive reporter polypeptide in said second fusion protein.

84. The method of Claim 74, wherein said first test polypeptide is fused to the N-terminus of said first inactive reporter polypeptide in said first fusion protein, and said second test polypeptide is fused to the C-terminus of said second inactive reporter polypeptide in said second fusion protein.

85. The method of Claim 74, wherein said active reporter protein is a counterselectable repoter.

86. The method of Claim 85, wherein said active reporter protein is a protein that directly or indirectly inhibits the host cell growth.

87. The method of Claim 85, wherein the expression of at least one of said fusion proteins in said host cell is inducible and occurs only when said host cell is subject to a predetermined condition.

88. The method of Claim 74, wherein said active reporter protein is a transcription activator and said host cell further comprises a detectable gene that is activated only when said transcription activator is present.

89. The method of Claim 74, wherein said active reporter protein is a transcription suppressor and said host cell further comprises a detectable gene that is suppressed only when said transcription suppressor is present.

90. The method of Claim 74, further comprising expressing a third test polypeptide in said host cell, wherein the interaction between said first and second test polypeptide requires the presence of said third test polypeptide.

91. The method of Claim 90, wherein said third test polypeptide modifies post-translationally at least one of said first and second test polypeptides.

92. The method of Claim 74, wherein each of said first and second fusion proteins further includes a membrane anchoring domain such that said first and second test polypeptides are exposed to an extracellular environment while anchored on cell membrane, and said N-intein, C-intein, and said first and second inactive reporter polypeptides are retained within the host cell.

93. A kit comprising:

a first expression vector containing a first chimeric gene having from 5' to 3'operably linked in the same open reading frame: (a) a sequence encoding a first inactive reporter polypeptide; (b) a coding sequence for N-intein; and (c) a first multiple cloning site; and

a second expression vector containing a second chimeric gene having from 5' to 3'operably linked in the same open reading frame: (a) a second multiple cloning site; (b) a coding sequencing for C-intein; (c) a sequence encoding a second inactive reporter polypeptide, wherein ligation between the C-terminus of said first inactive reporter polypeptide and the N-terminus of said second inactive reporter polypeptide forms an active reporter.

94. The kit of Claim 93, wherein said N-intein do not interact with said C-intein.

95. The kit of Claim 93, wherein each of said first and second chimeric genes further comprises a sequence encoding a membrane anchoring domain.

96. The kit of Claim 93, further comprising a third expression vector containing a DNA sequence encoding an enzyme capable of modifying proteins *in vivo*.

97. A kit comprising:

an expression vector containing a chimeric gene having from 5' to 3'operably linked in the same open reading frame: (a) a sequence encoding a first inactive reporter polypeptide; (b) a coding sequence of an N-intein or C-intein; and (c) a first multiple cloning site; and

an expression library expressing a plurality of fusion proteins, each of said fusion proteins having from N-terminus to C-terminus: (a) a random polypeptide; (b) a C-intein or N-intein; and (c) a second inactive reporter polypeptide, wherein ligation between the C-terminus of said first inactive reporter polypeptide and the N-terminus of said second inactive reporter polypeptide forms an active reporter.

INTEIN-BASED METHOD FOR DETECTING PROTEIN-PROTEIN INTERACTIONS

Abstract

A method for detecting protein-protein interaction is provided, in which two intein-containing fusion proteins are prepared and allowed to interact with each other. One fusion protein includes an N-intein and a first test polypeptide, and the other fusion protein includes a C-intein and a second test polypeptide. One or both of the two fusion proteins have an inactive reporter capable of being converted to an active reporter upon trans-splicing mediated by the N-intein and the C-intein. The active reporter is detected which would indicate the interaction between the two test polypeptides. The system is also useful in characterizing protein-protein interactions and identifying compounds that modulate particular protein-protein interactions.

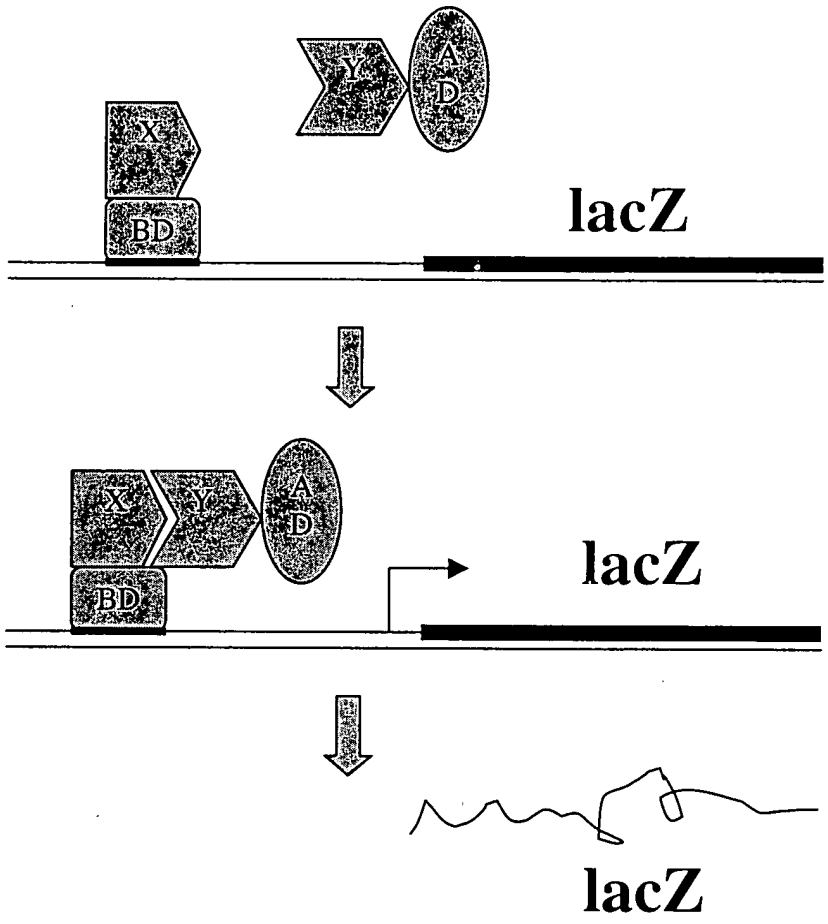

FIGURE 1 (Prior Art)

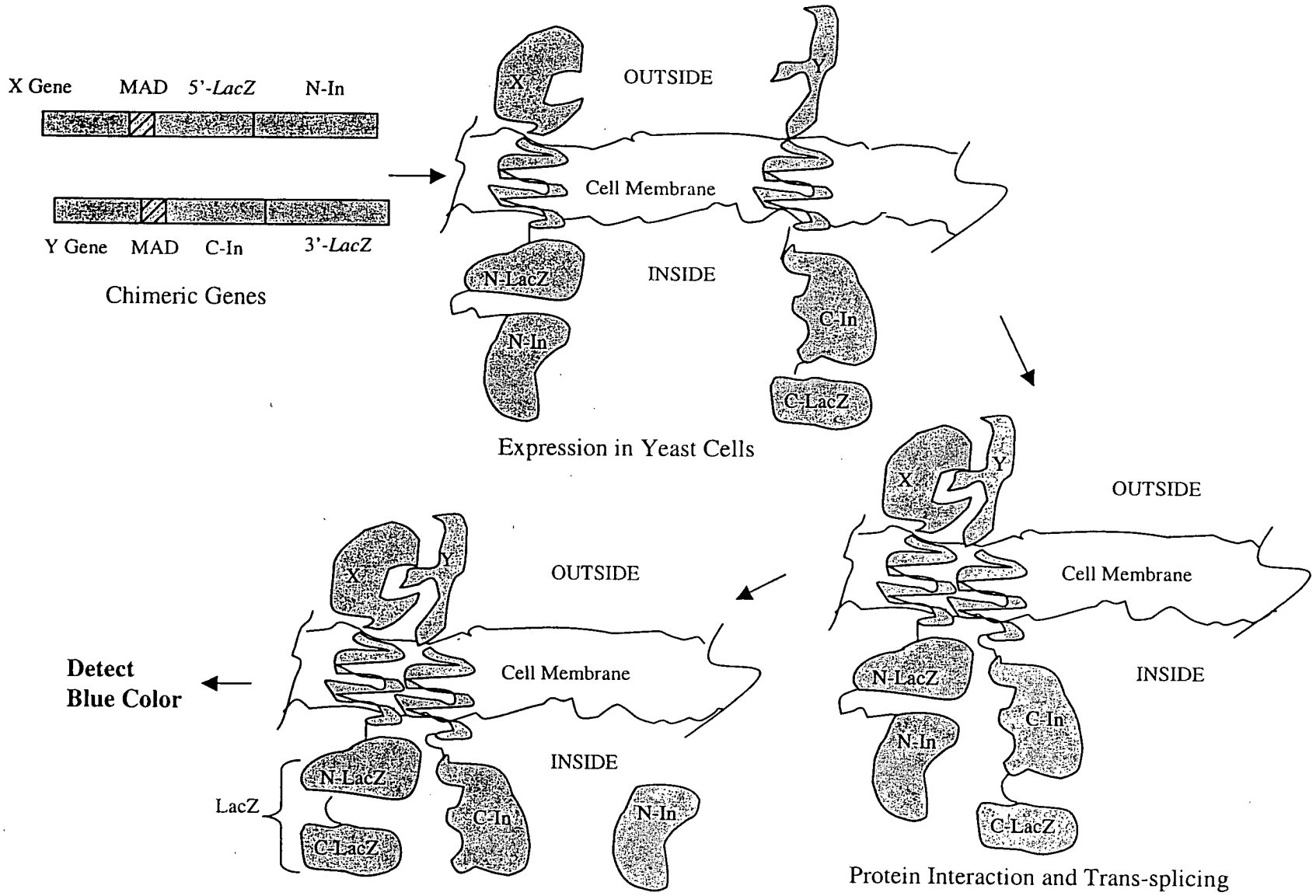

Figure 7 Detecting Extracellular Protein-Protein Interaction

EXHIBIT E

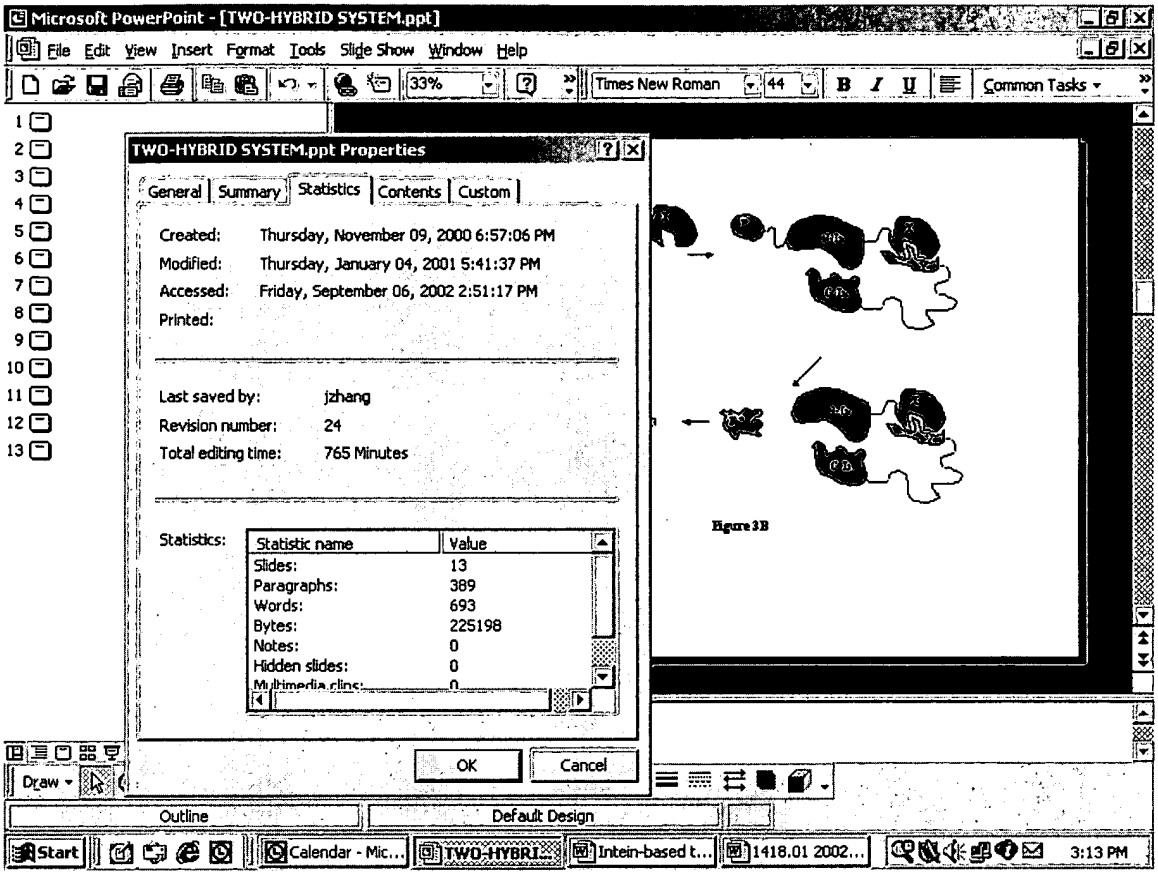
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EXHIBIT F

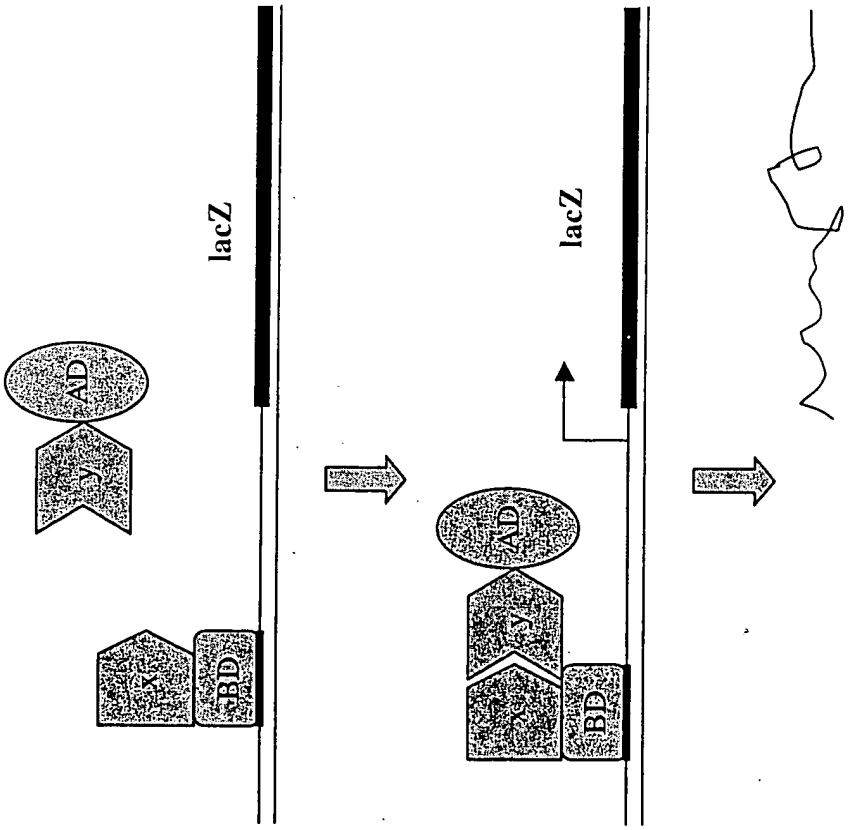


FIGURE 1 (Prior Art)

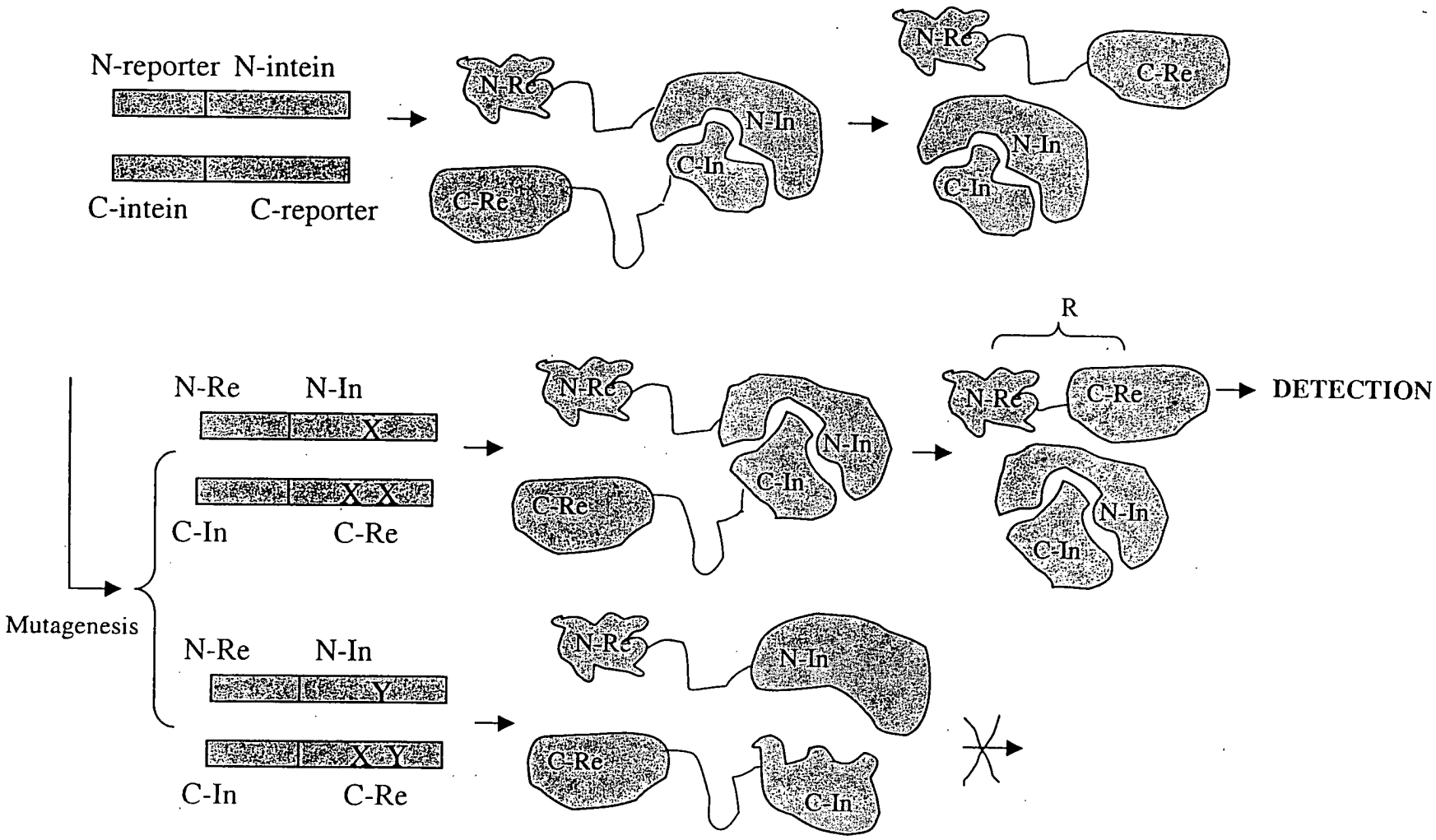


Figure 2A Selecting Non-interacting N-intein & C-intein

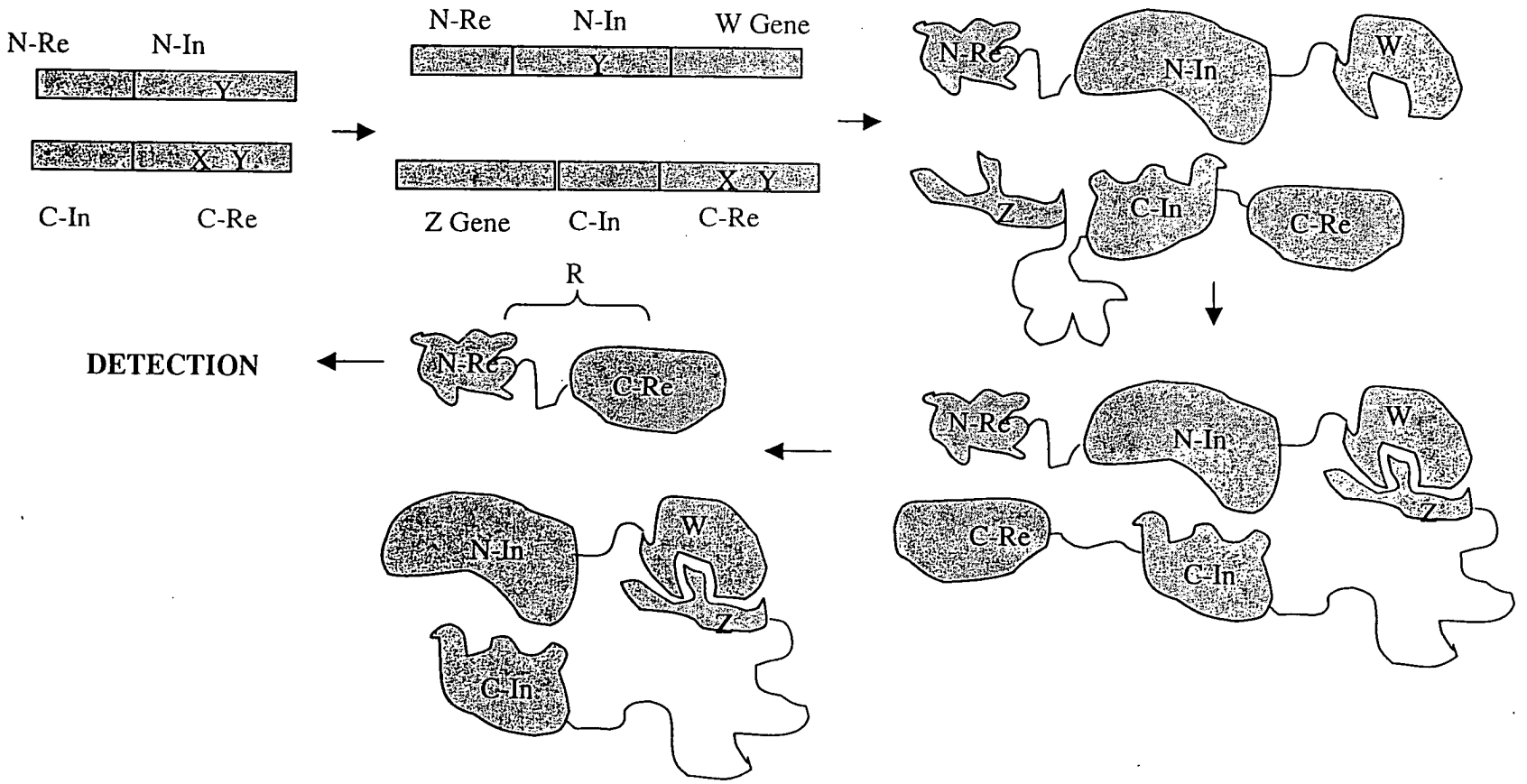


Figure 2B Verifying That the Selected Non-interacting N-intein & C-intein Are Capable of Mediating Protein Splicing

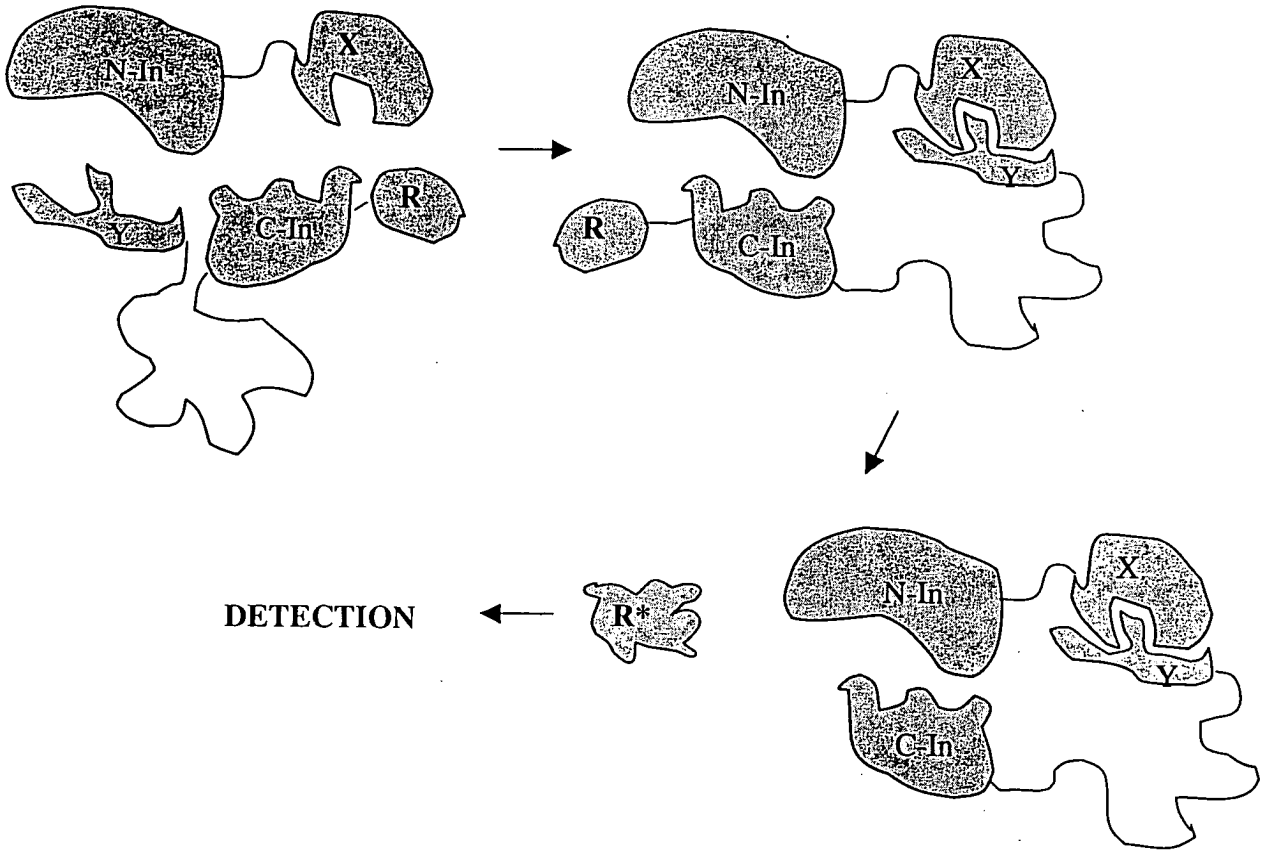


Figure 3A

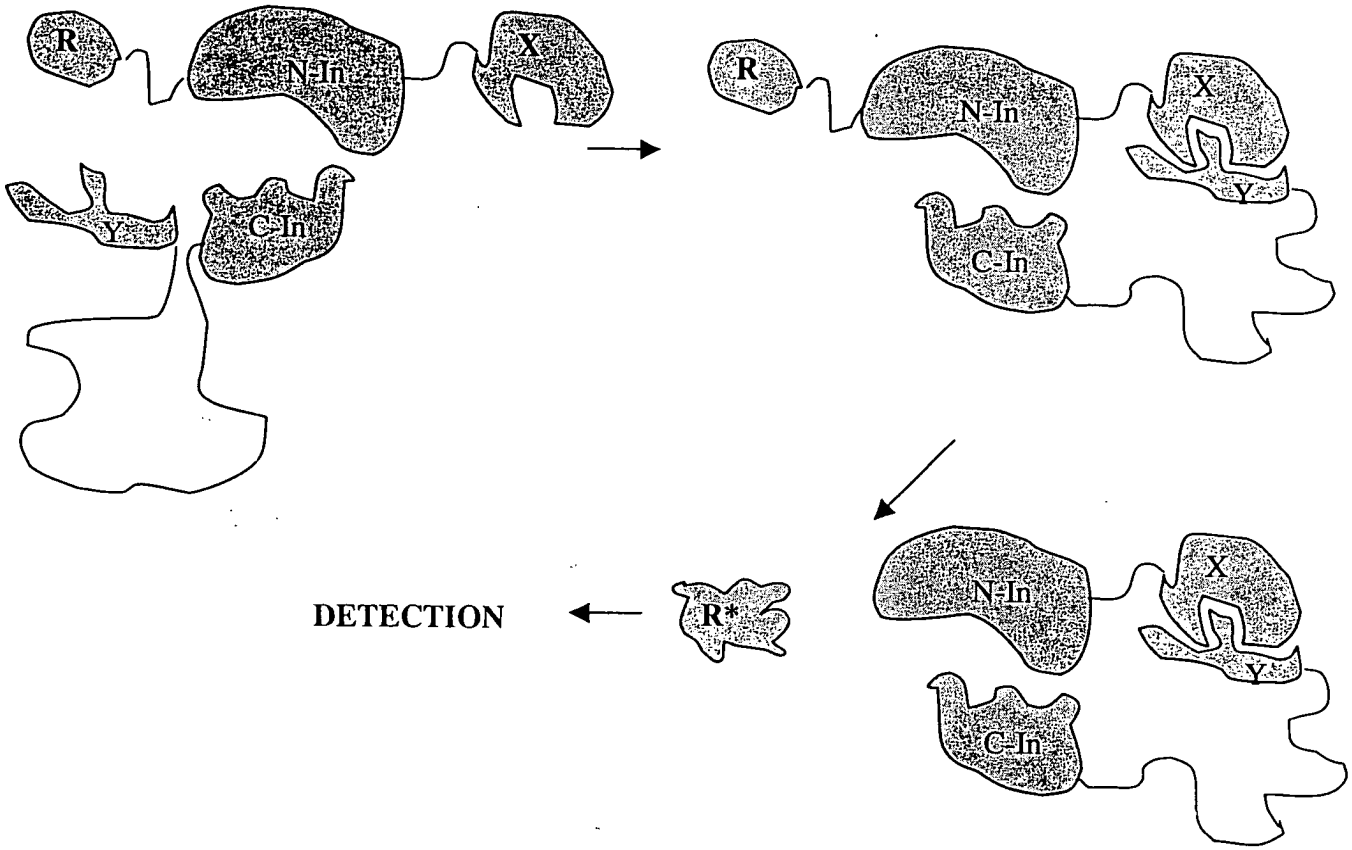


Figure 3B

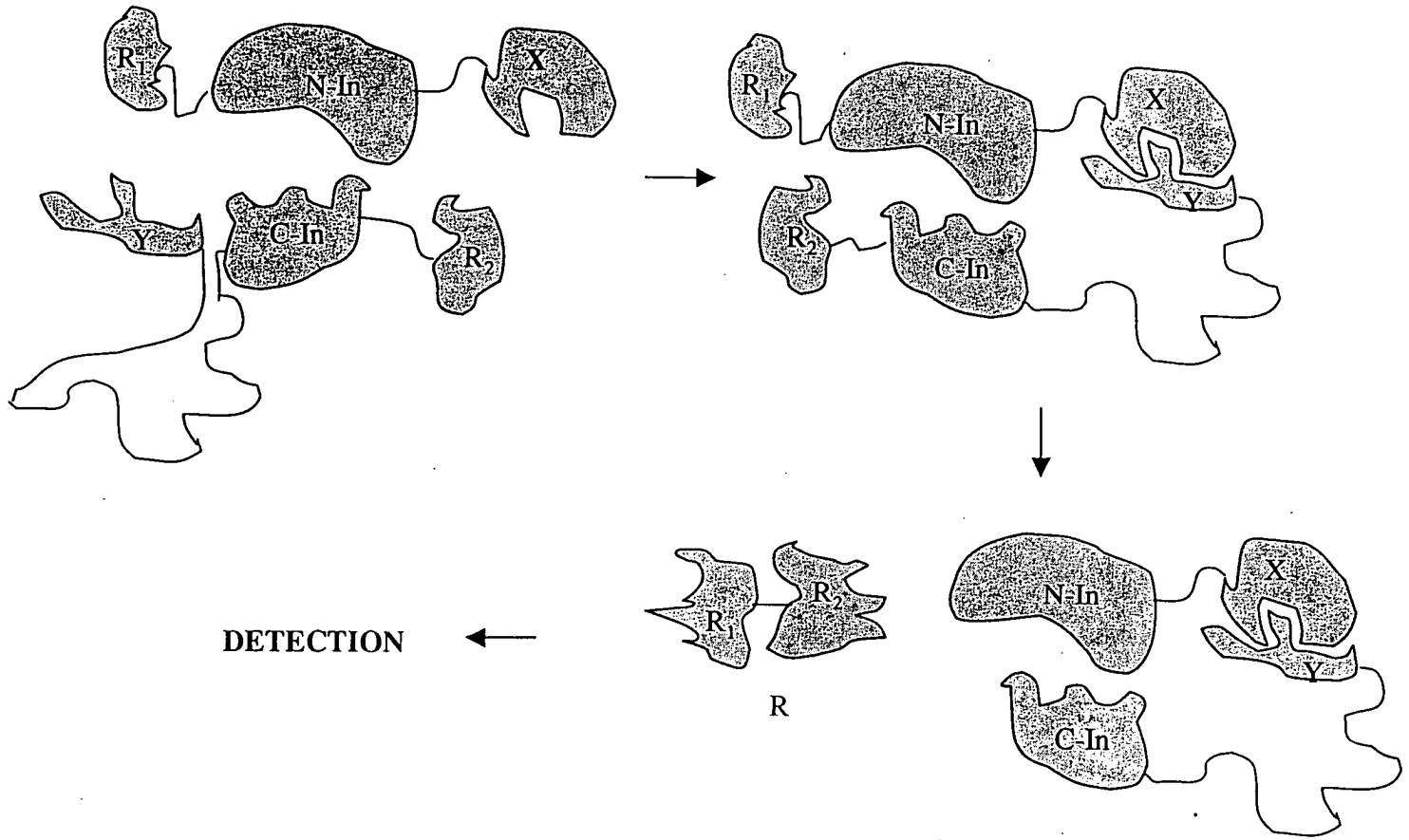


Figure 3C

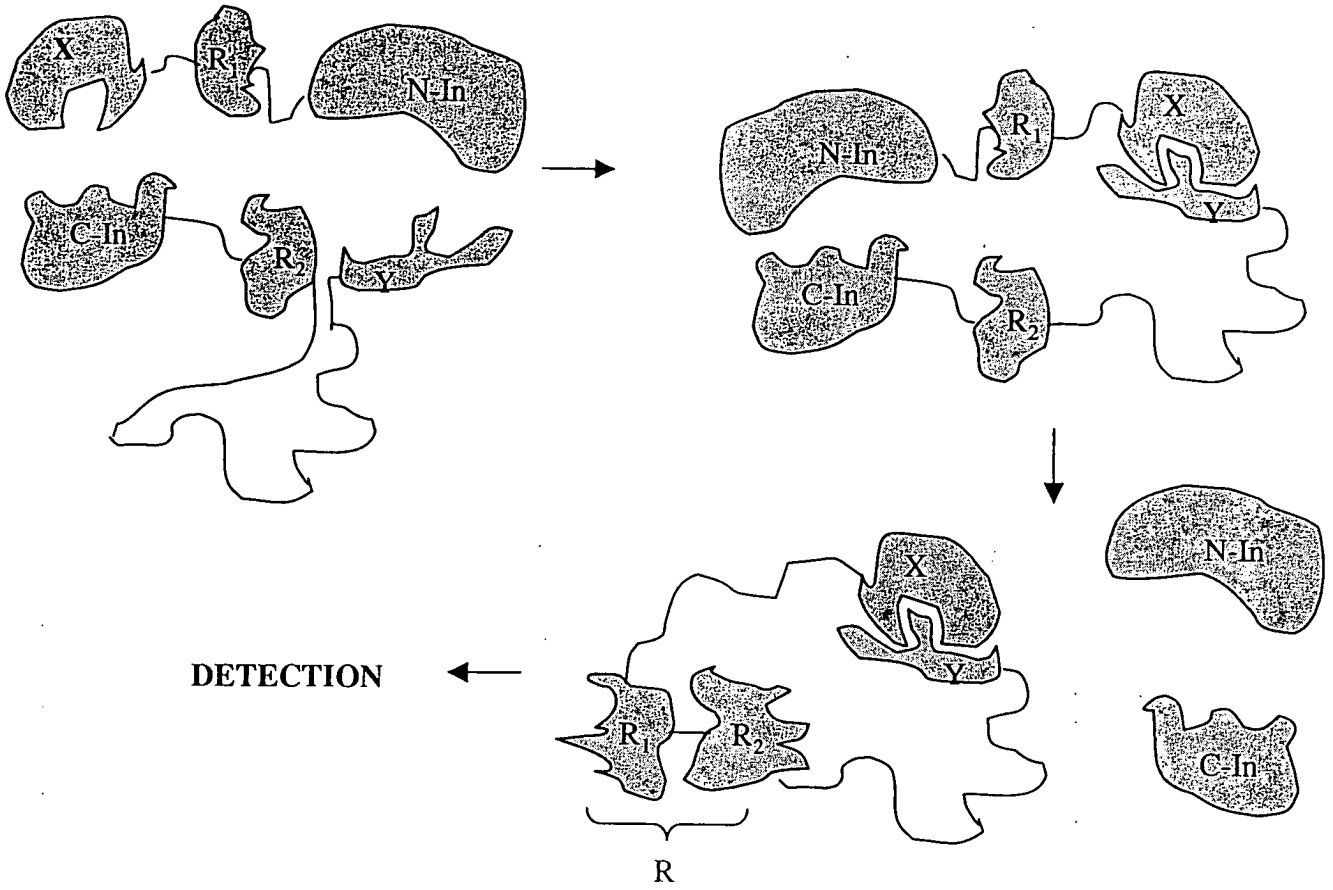


Figure 3D

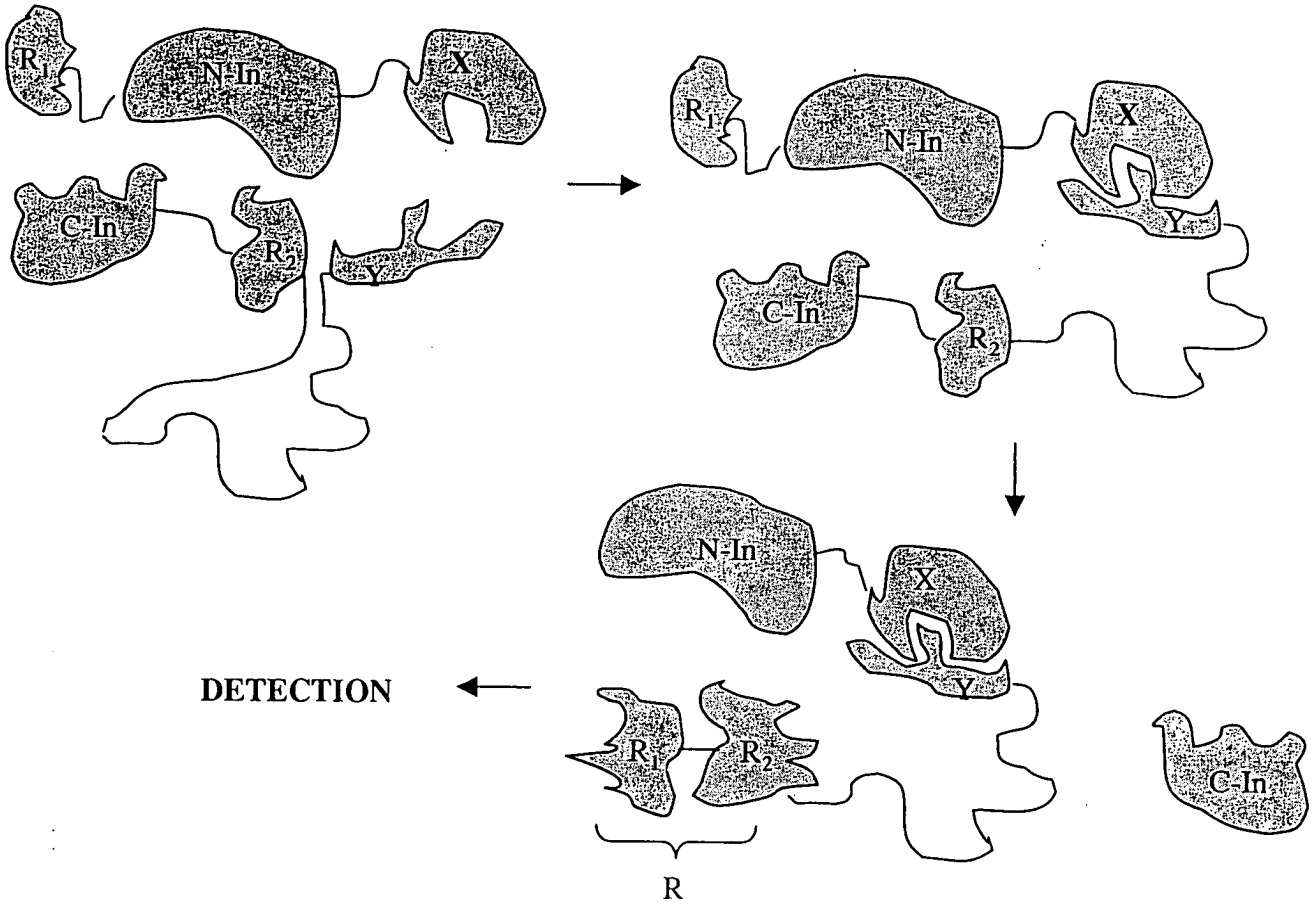


Figure 3E

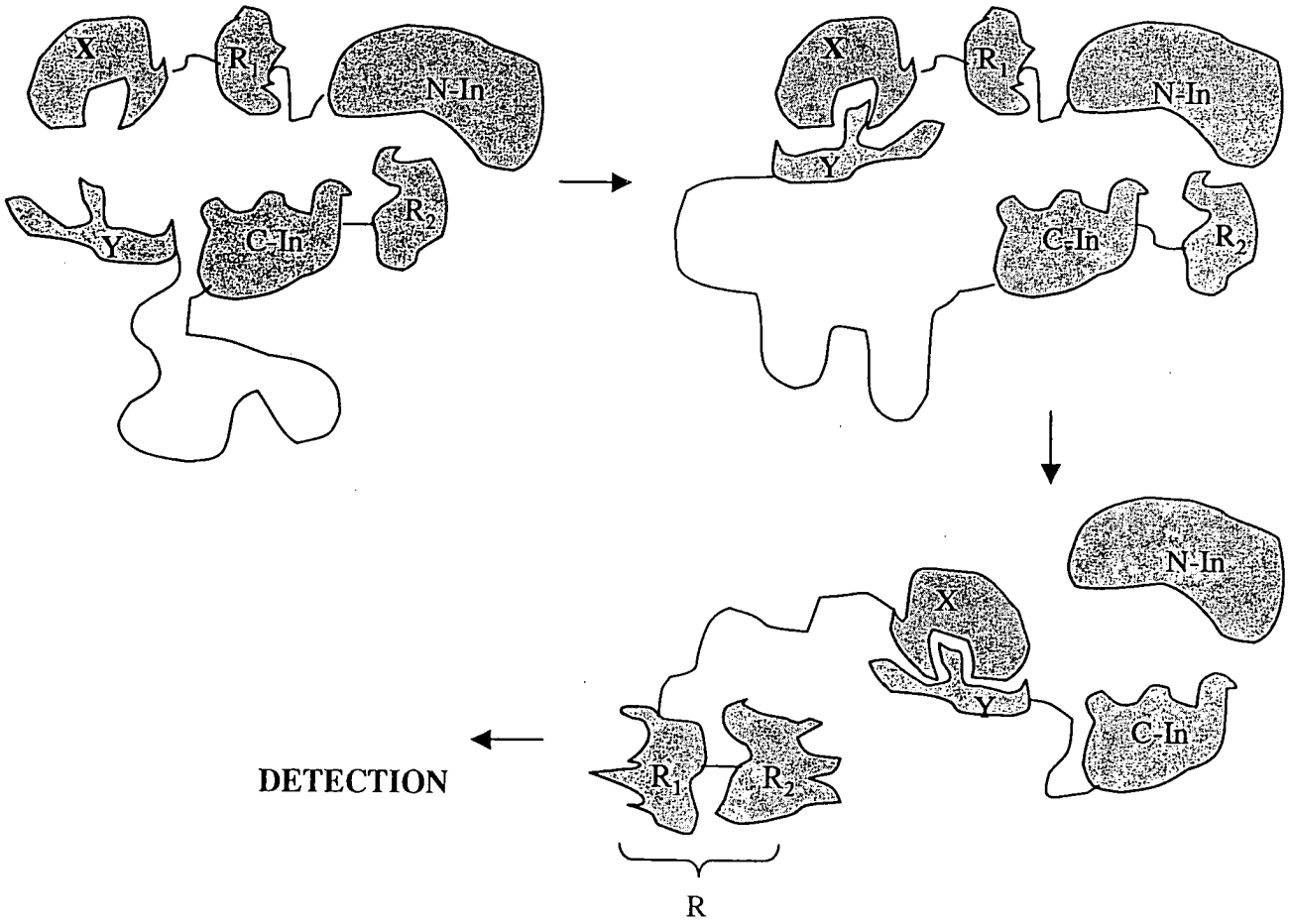
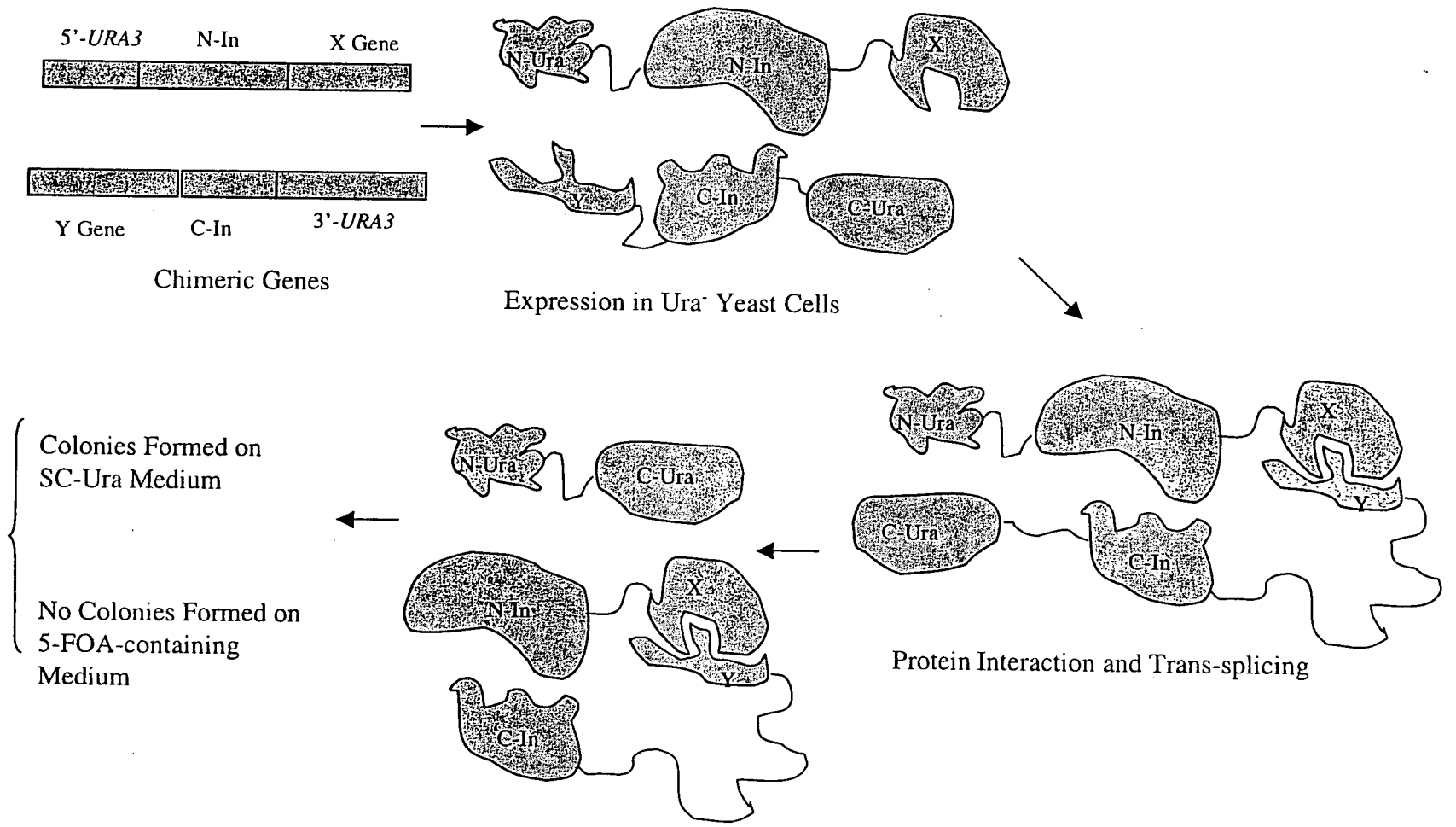


Figure 3F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X Gene
Y Gene
C-In
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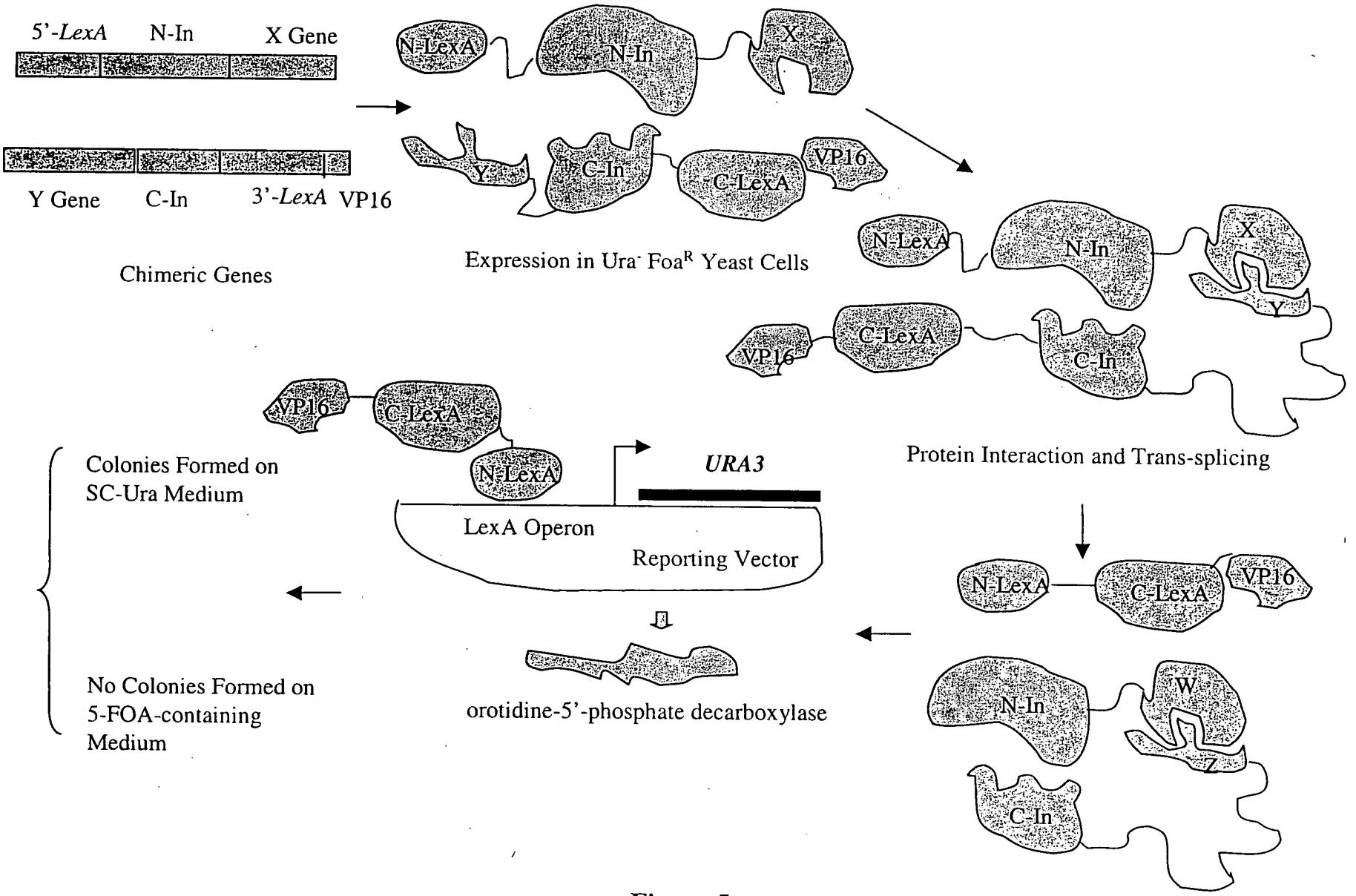
N-In
X
Y
C-In
C-Ura
Expression in Ura- Yeast Cells
Protein Interaction and Trans-splicing
Colonies Formed on SC-Ura Medium
No Colonies Formed on 5-FOA-containing Medium

Figure 4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Reporting Vector
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Colonies Formed on SC-Ura Medium
No Colonies Formed on 5-FOA-containing Medium
W
Z

Figure 5

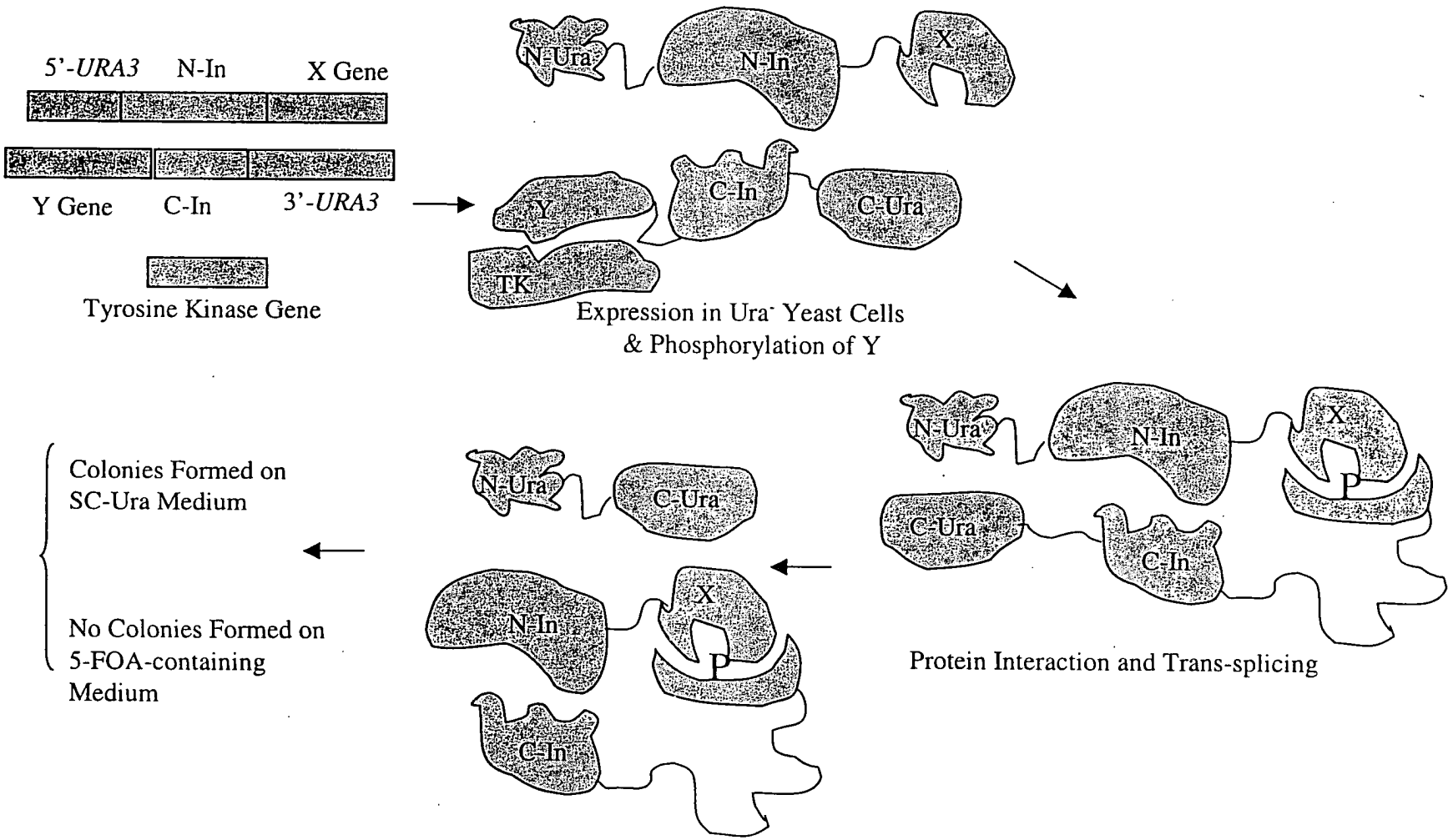

Figure 6 Intein-Based Multi-Hybrid System